

**Activation of innate immunity
during systemic *Candida* infections**

Daniela Camelia van 't Ent - Ifrim

Colofon

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Pentru părinții mei

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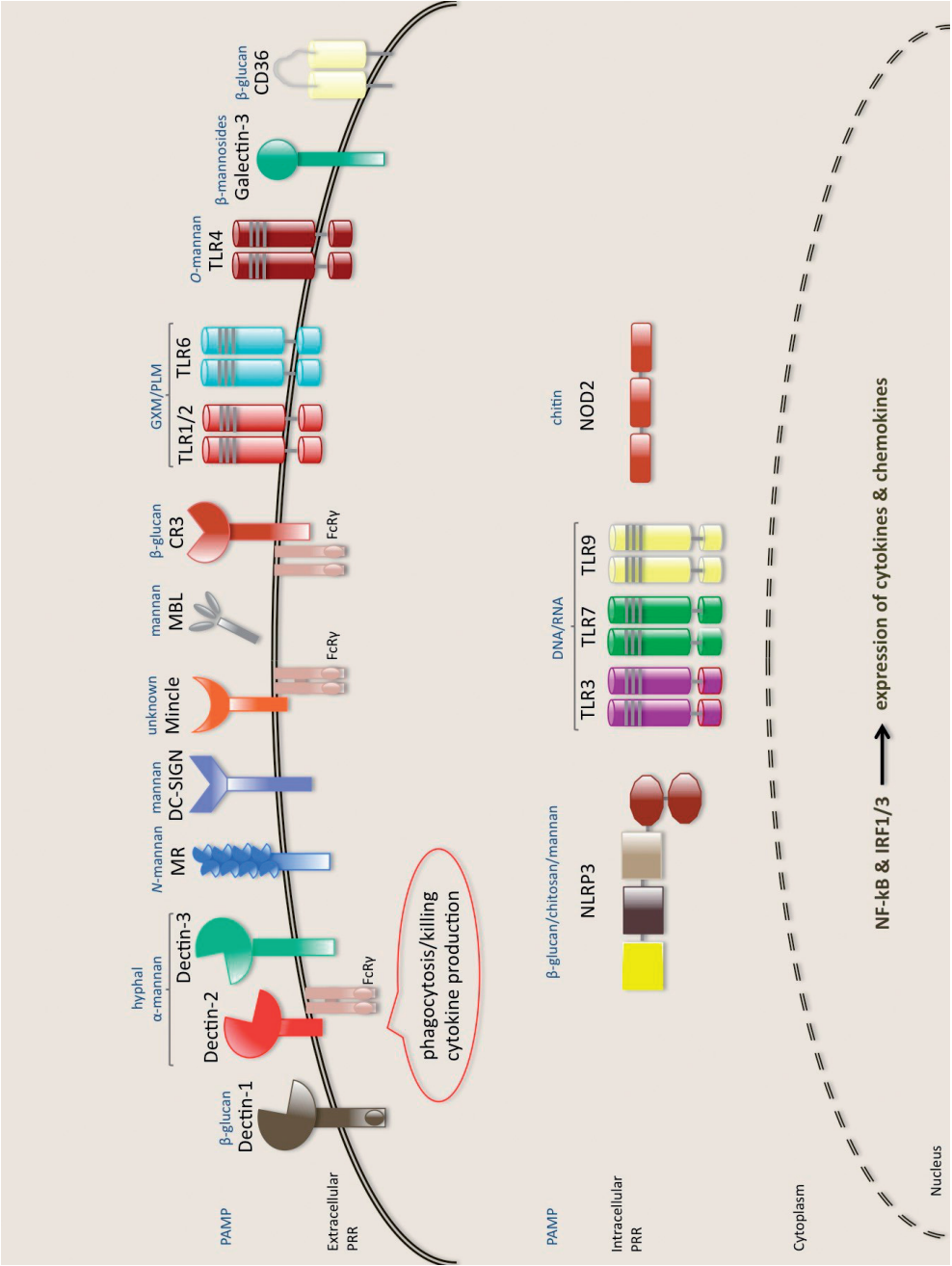
General introduction and outline of the thesis



The gastrointestinal tract is colonized by a distinct array of microbes that is specific to each individual (1). Whereas the human body consists of trillions somatic cells, it harbors a larger number of microorganisms in the intestines (2-4). In general, human commensals exhibit beneficial effects for the host; however, gut microorganisms may also be deleterious, as for instance in immunocompromised hosts where they invade the tissues, leading to mucosal and disseminated infection. Hence, the immune system plays a significant role in keeping the pathogens at bay. While some of the fungi are non-harmful commensals, others may cause mild to severe cutaneous infections or even life-threatening systemic infections. Whereas the total number of eukaryotic species on Earth has been estimated at roughly 9 million, with fungi representing approximately 7% of this number (5), only about 600 species of fungi are human pathogens (6). *Candida spp* are the fourth most common cause of hospital-acquired systemic infections in the United States with crude mortality rates of up to 50% (7, 8).

During the course of fungal infection, one of the first steps in mounting effective immune responses is the recognition of the invaders by the host, with Pattern Recognition Receptors (PRRs) as key players in this phenomenon. Numerous studies revealed differential roles of receptors present on/within the immune cells, PRRs that are classified in four families: C-type lectin-like (CLR), Toll-like (TLR), NOD-like (NLR), and RIG-I-like (RLR) receptors. Most known PRRs that bind to *Candida* Pathogen Associated Molecular Patterns (PAMPs) are C-type lectins. Mannan present in the fungal cell wall is mainly recognised by Dectin-2 (9), Mannose Receptor (10), Galectin-3 (11), DC-SIGN (12) and MBL (13), recognition that leads to the induction of protective proinflammatory cytokines. Dectin-1 recognizes β -1,3 and β -1,6-glucan on *Candida* conidia, resulting in the subsequent induction of cytokines and fungus internalisation (14, 15). The majority of CLRs signal via Syk/CARD9 (16, 17), while DC-SIGN activates NF- κ B subunit p56 via Raf1 (18). TLR2 and TLR4, which signal through MyD88, bind to peptidoglycan and play a significant role in *Candida* recognition as shown by the experiments conducted with genetically modified mice (19-22). Chitin is a component of the inner cell wall of fungi and induces IL-10 and TNF- α through MR, TLR2 and Dectin-1 mediated signalling pathways, with NOD2 and TLR9 required for its recognition (23, 24). NLRP3 is important in controlling mucosal and disseminated candidiasis by processing pro-IL-1 β to IL-1 β downstream of Dectin-1 and TLR2, and mice deficient in NLRP3 are more susceptible to *Candida* infection (25-27). Nevertheless, the specific impact of several other PRRs that possibly contribute to antifungal host defense remains unknown, and, especially differences in recognition of various *Candida* species are poorly investigated.

In the first part of the thesis, I will investigate the specific roles of two of the most important C-type lectin receptors, Dectin-1 and Dectin-2, for the recognition of *Candida* and the induction of effective innate immune responses. In **Chapter 2** the *in vitro* priming effects of *Candida albicans* in human Peripheral Blood Mononuclear Cells (PBMCs) will be explored. Moreover, cells deficient in Dectin-1 receptor and a number of *Candida* cell wall mutants will be evaluated in order to identify which *Candida* component and which signaling pathway is responsible for the priming. In **Chapter 3** the biological role of Dectin-2 receptor in interaction with *Candida albicans* will be evaluated, whereas **Chapter 4** will



- ◀ **Figure 1:** Recognition of *C. albicans*. The pattern recognition receptors present on the immune cells recognize the fungal cell wall components and intracellular DNA and RNA. These signals activate canonical or non-canonical nuclear pathways, with the subsequent induction of the immune responses, such as secretion of cytokines, chemokines, initiation of phagocytosis and/or inflammasome activation. Adaptation from 'Antifungal innate immunity: recognition and inflammatory networks' in *Seminars in Immunopathology* by Becker & Ifrim, 2014.

reveal the effects of systemic candidiasis caused by *Candida glabrata* in murine models, and, particularly the contribution of Dectin-2 to antifungal defense.

One of the dogma of immunology is that innate immune responses are only temporarily activated during an infection, after which they return to normal: the innate immune response would thus react identically each time it encounters a pathogen. That would, therefore, by definition exclude the presence of adaptive characteristics, or immunological memory, within the innate immune system. Subsequently, this fact would lead to the conclusion that solely vertebrates have immunological memory, as all other organisms on Earth have only innate host defense. The hypothesis that the innate immune system is incapable of mounting adaptive responses (28) has been however contradicted by studies showing that organisms lacking adaptive immune system, such as plants and insects, have specific memory and are able to mount a response to a secondary infection (29). Furthermore, mice lacking T- and B-cells were

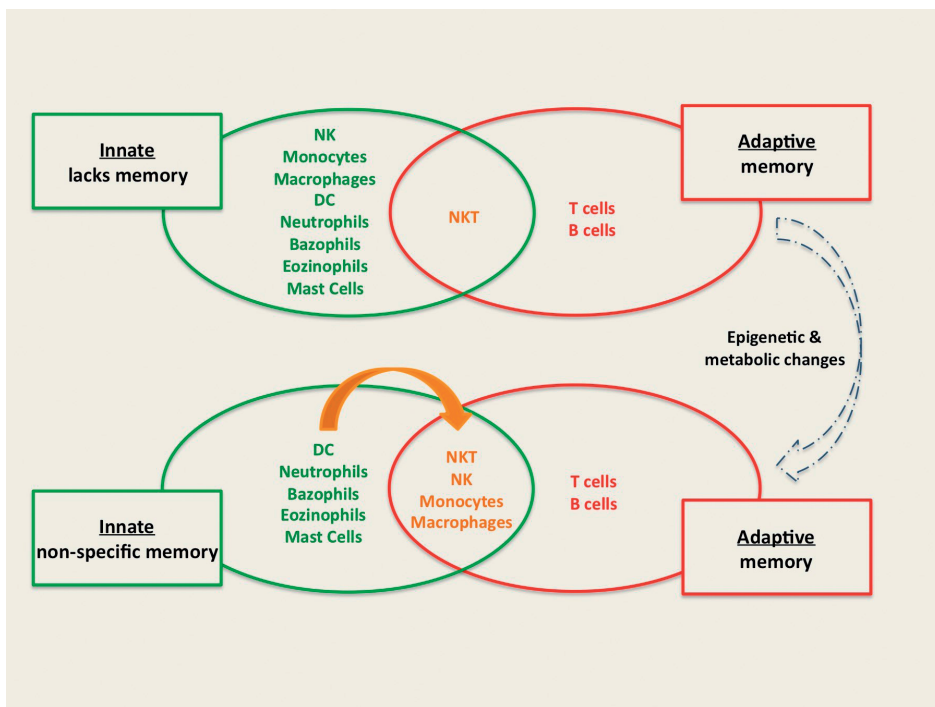


Figure 2: Trained immunity

protected by microbial infections, protection offered by NK cells and monocytes, respectively (30, 31). Therefore, the immune cells such as NK cells and monocytes/macrophages are able to exhibit adaptive characteristics (32), with epigenetic reprogramming of monocytes at the level of histone H3 methylation as the molecular mechanism responsible for long-term memory of innate immunity: this process has been termed ‘*trained immunity*’ (31, 33, 34).

Subsequently, the initiation of innate immune memory through trained immunity might be responsible on one hand for the nonspecific protective effects of certain vaccines, such as well-known Bacille Calmette-Guerin (BCG) (35), and on the other hand for excessive inflammatory responses in autoinflammatory/autoimmune diseases (36). Therefore, the effects of trained immunity phenomena can be explored at different levels, either by identifying potential therapeutic targets for excessive inflammatory diseases, or by assessing long-term responses of monocytes, macrophages and NK cells to a large spectrum of microbes that confer resistance or tolerance to subsequent infections (34, 37-41).

In the second part of the thesis I will investigate the capacity of *Candida* to induce innate immune memory after a challenge (or infection). In **Chapter 5** the training effect of different ligands, present either at the level of the cell wall or inside the cell of regular commensals of human flora, will be assessed. Their effects on primary monocytes and the impact on our knowledge regarding the complex interactions among the immune cells and different microbes will be described. The immune reactivity of *S. cerevisiae* at a strain level and its ability to modulate the cytokine responses of human primary cells to bacteria and fungi will be addressed in **Chapter 6**. In addition, in **Chapter 7** we will study the capacity of *Candida albicans* to train primary cells from Chronic Mucocutaneous Candidiasis (CMC) and Hyperimmunoglobulin E Syndrome (HIES) patients, with an emphasis on NK cells and IFN- γ .

In order to present an up-to-date overview of our knowledge regarding the pattern recognition receptors and inflammatory networks of two very common fungal pathogens, *Candida albicans* and *Aspergillus fumigatus*, a bibliographic review will be provided in **Chapter 8**. Finally, an overall summary of the findings presented in this thesis will be described in **Chapter 9**. In this chapter I will propose future directions for the increased understanding of the role of C-type lectin receptors in *Candida* infections and I will point out likely challenging prospects with regard to training the immunity.

References

- 1 Forssten SD, Sindelar CW, Ouwehand AC. Probiotics from an industrial perspective. *Anaerobe*. 2011 Dec;17(6):410-3.
- 2 Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol*. 2001 Oct;108(4):516-20.
- 3 O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep*. 2006 Jul;7(7):688-93.
- 4 Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. 2012 Jun 8;336(6086):1314-7.
- 5 Mora C, Tittensor DP, Adl S, Simpson AG, Worm B. How many species are there on Earth and in the ocean? *PLoS Biol*. 2011 Aug;9(8):e1001127.
- 6 Brown GD, Denning DW, Levitz SM. Tackling human fungal infections. *Science*. 2012 May 11;336(6082):647.
- 7 Pfaller MA, Diekema DJ. Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol*. 2010;36(1):1-53.
- 8 Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev*. 2007 Jan;20(1):133-63.
- 9 Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity*. 2010 May 28;32(5):681-91.
- 10 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol*. 2008 Jan;6(1):67-78.
- 11 Jouault T, El Abed-El Behi M, Martinez-Esparza M, Breuilh L, Trinel PA, Chamaillard M, et al. Specific recognition of *Candida albicans* by macrophages requires galectin-3 to discriminate *Saccharomyces cerevisiae* and needs association with TLR2 for signaling. *J Immunol*. 2006 Oct 1;177(7):4679-87.
- 12 Cambi A, Netea MG, Mora-Montes HM, Gow NA, Hato SV, Lowman DW, et al. Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J Biol Chem*. 2008 Jul 18;283(29):20590-9.
- 13 Saevarsdottir S, Vikingsdottir T, Valdimarsson H. The potential role of mannan-binding lectin in the clearance of self-components including immune complexes. *Scand J Immunol*. 2004 Jul-Aug;60(1-2):23-9.
- 14 Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, et al. Dectin-1 is a major beta-glucan receptor on macrophages. *J Exp Med*. 2002 Aug 5;196(3):407-12.
- 15 Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, et al. Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. *Nature*. 2011 Apr 28;472(7344):471-5.
- 16 Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol*. 2009 Jul;9(7):465-79.
- 17 Kerscher B, Willment JA, Brown GD. The Dectin-2 family of C-type lectin-like receptors: an update. *Int Immunol*. 2013 May;25(5):271-7.
- 18 Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity*. 2007 May;26(5):605-16.
- 19 Villamon E, Gozalbo D, Roig P, O'Connor JE, Ferrandiz ML, Fradelizi D, et al. Toll-like receptor 2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model of disseminated candidiasis. *Microbes Infect*. 2004 May;6(6):542-8.
- 20 Netea MG, Suttmuller R, Hermann C, Van der Graaf CA, Van der Meer JW, van Krieken JH, et al. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol*. 2004 Mar 15;172(6):3712-8.
- 21 Netea MG, Van Der Graaf CA, Vonk AG, Verschuere I, Van Der Meer JW, Kullberg BJ. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis*. 2002 May 15;185(10):1483-9.
- 22 Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, et al. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol*. 2004 Mar 1;172(5):3059-69.
- 23 Wagener J, Malireddi RK, Lenardon MD, Koberle M, Vautier S, MacCallum DM, et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog*. 2014 Apr;10(4):e1004050.

- 24 Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J Immunol*. 2009 Mar 15;182(6):3573-82.
- 25 Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med*. 2009 Aug 31;206(9):2037-51.
- 26 Gross O, Poeck H, Bscheider M, Dostert C, Hanneschlager N, Endres S, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. 2009 May 21;459(7245):433-6.
- 27 Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, et al. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. *Cell Host Microbe*. 2009 May 8;5(5):487-97.
- 28 Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.
- 29 Kurtz J. Specific memory within innate immune systems. *Trends Immunol*. 2005 Apr;26(4):186-92.
- 30 Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. 2009 Jan 29;457(7229):557-61.
- 31 Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A*. 2012 Oct 23;109(43):17537-42.
- 32 Bowdish DM, Loffredo MS, Mukhopadhyay S, Mantovani A, Gordon S. Macrophage receptors implicated in the "adaptive" form of innate immunity. *Microbes Infect*. 2007 Nov-Dec;9(14-15):1680-7.
- 33 Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe*. 2012 Aug 16;12(2):223-32.
- 34 Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirofeh A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014 Sep 26;345(6204):1251086.
- 35 Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. *Trends Immunol*. 2013 Sep;34(9):431-9.
- 36 Bekkering S, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Trained innate immunity and atherosclerosis. *Curr Opin Lipidol*. 2013 Dec;24(6):487-92.
- 37 Jaskiewicz M, Conrath U, Peterhansel C. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep*. 2011 Jan;12(1):50-5.
- 38 Brown GD, Gordon S. Fungal beta-glucans and mammalian immunity. *Immunity*. 2003 Sep;19(3):311-5.
- 39 Oren R, Farnham AE, Saito K, Milofsky E, Karnovsky ML. Metabolic patterns in three types of phagocytizing cells. *J Cell Biol*. 1963 Jun;17:487-501.
- 40 O'Neill LA, Hardie DG. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature*. 2013 Jan 17;493(7432):346-55.
- 41 Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol*. 2014 Oct 25;155(2):213-9.

PART ONE

Innate immune recognition by C-type lectin receptors

2

***Candida albicans* Primes TLR Cytokine Responses through a Dectin-1/Raf-1–Mediated Pathway**

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Abstract

The immune system is essential to maintain homeostasis with resident microbial populations, ensuring that the symbiotic host–microbial relationship is maintained. In parallel, commensal microbes significantly shape mammalian immunity at the host mucosal surface, as well as systemically. *Candida albicans* is an opportunistic pathogen that lives as a commensal on skin and mucosa of healthy individuals. Little is known about its capacity to modulate responses toward other microorganisms, such as colonizing bacteria (e.g., intestinal microorganisms). The aim of this study was to assess the cytokine production of PBMCs induced by commensal bacteria when these cells were primed by *C. albicans*. We show that *C. albicans* and β -1,3-glucan induce priming of human primary mononuclear cells and this leads to enhanced cytokine production upon *in vitro* stimulation with TLR ligands and bacterial commensals. This priming requires the β -1,3-glucan receptor dectin-1 and the noncanonical Raf-1 pathway. In addition, although purified mannans cannot solely mediate the priming, the presence of mannosyl residues in the cell wall of *C. albicans* is nevertheless required. In conclusion, *C. albicans* is able to modify cytokine responses to TLR ligands and colonizing bacteria, which is likely to impact the inflammatory reaction during mucosal diseases.

Keywords: *Candida*, priming, Dectin-1/Raf-1

Introduction

Immediately after birth, the gastrointestinal tract is colonized by a diverse array of microbes, leading to a stable microbiota that is unique to each individual (1). The human body consists of trillions of somatic cells and harbors about 10 times more microorganisms in the intestines (2). The adult microbiota is composed of permanent members (up to 1000 species), as well as transient colonizers that are acquired from external sources. It consists of many prokaryotic and eukaryotic microbes, but bacterial species dominate. The most common anaerobic bacteria within the gastrointestinal tract are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium*, and *Lactobacillus* species (3). These organisms are present in extremely high concentrations and have evolved to degrade a variety of dietary substances, simultaneously enhancing the host digestive efficiency, as well as their own nutrient supply (4). In addition to their beneficial effects, gut microorganisms may be deleterious: when host defense is compromised, they may invade host tissues, which leads to mucosal and sometimes subsequent disseminated infection. A role for the gut flora in the pathogenesis of autoimmune diseases, such as Crohn's disease and ulcerative colitis, has also long been suggested (5). Hence, the immune system controls microbial composition; conversely, the gut microflora shapes immunity (6). Commensal microbes influence mucosal and systemic immunity, lymphoid structure development and epithelial function, T cell subsets, and innate lymph cells (6). *Candida albicans* is primarily a human commensal of the skin and mucous membranes; however, under circumstances of disturbed host defense, it may become a pathogen (7, 8). The dual consequences of *C. albicans* colonization and/or infection suggest that this fungus might modulate the host immune response against other colonizing microorganisms. It was demonstrated that *C. albicans* triggers differential immune signaling upon interaction with either inflammatory or tolerogenic dendritic cells (9). The role of *C. albicans* in experimental colitis is controversial: on one hand it may activate dendritic cells in Peyer's patches (9), whereas on the other hand it may increase the severity of colitis (10, 11). Nevertheless, all of these studies were performed in mice, and it is not known whether *Candida* modulates inflammation in human primary cells.

In this study, using human primary cells, we investigated whether *C. albicans* is able to modulate cytokine responses to secondary stimuli, such as TLR ligands and colonizing microorganisms, by inducing either inflammation or tolerance. We established that *C. albicans* and, more specifically, β -1,3-glucan (β -glucan) can efficiently prime human immune cell responses to TLRs and bacteria, leading to enhanced cytokine production *in vitro*. By deciphering the receptor-signaling pathway involved (dectin-1/Raf-1), we also provide a possible novel therapeutic target in autoimmune disorders.

Materials and Methods

Blood samples. Blood was collected from healthy volunteers at Sanquin Bloodbank in Nijmegen, The Netherlands, after obtaining informed consent. Human PBMCs were isolated from buffy coats.

Reagents. Ficoll-Paque (GE Healthcare) was used to isolate PBMCs by differential centrifugation. RPMI 1640 Dutch Modification (RPMI) (Sigma-Aldrich), supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate (Life Technologies, Nieuwekerk, The Netherlands) was used as culture medium. *Candida* β -glucan and mannan were isolated and purified as described previously (12, 13). Three sources of *C. albicans* mannan were tested: mannan serotype A, mannan isolated from *Candida* yeast grown in yeast extract/peptone/dextrose broth at 30°C (pronase treated), and mannan isolated from *Candida* hyphae grown in serum at 37°C (not treated with pronase). Pam3Cys4 (EMC microcollections) and LPS (*Escherichia coli* serotype 055:B5) were purchased from Sigma-Aldrich, with an additional purification step for LPS (14). Human dectin-1/CLEC7A mAb and isotype Ab were purchased from R&D Systems (Abingdon, U.K.).

Microorganisms. *Candida albicans* ATCC MYA-3573 (UC 820), *och1* Δ , *och1* Δ /*och1* Δ /*OCH1*, *pmr1* Δ , *pmr1* Δ /*pmr1* Δ /*PMR1*, *mnn4* Δ , *mnn4* Δ /*mnn4* Δ /*MNN4*, and the parental strain *C. albicans* NGY152 were grown overnight to yeast cells in Sabouraud broth at 37°C. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in culture medium (RPMI; ICN Biomedicals, Aurora, OH). *Candida* yeast was heat-killed for 30 min at 95°C. To generate hyphae, yeast cells were grown at 37°C in culture medium adjusted to pH 6.4 using hydrochloric acid. Hyphae were killed by exposure to 95°C for 30 min and resuspended in culture medium to a hyphal inoculum size that originated from 1x10⁶/ml. The *OCH1* gene was disrupted using the ura-blaster method (15), and the reintegrant strain was constructed as previously described (16). Creation of the *mnn4* null mutant and of its reintegrant *mnn4* Δ /*mnn4* Δ /*MNN4* control strain was performed as described (17). The *PMR1* gene was disrupted and reintroduced in *pmr1* Δ /*pmr1* Δ /*PMR1* as described (18). Three common colonizing bacteria of the intestines or the skin were chosen: *Escherichia coli* ATCC 35218 was grown overnight in culture medium, washed three times with PBS, and heat-killed for 60 min at 80°C; *Staphylococcus aureus* strain Seattle 1945 (ATCC 25923) was grown overnight in culture medium, washed twice with cold PBS, and heat-killed for 30 min at 100°C; and *Bacteroides fragilis* ATCC 25285 was grown as before but was heat-killed for 30 min at 95°C.

In vitro cytokine production. The PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood:1 part pyrogen-free saline) over Ficoll-Paque (GE Healthcare). PBMCs were washed twice in saline and resuspended in culture medium. Cells were counted with a Beckman Coulter Z1 Particle Counter and adjusted to 5x10⁶ cells/ml. PBMCs (5x10⁶) were added at a volume of 100 μ l/well in round-bottom 96-well plates. Cells were incubated with one of the first stimuli for 24 h and reincubated for another 24 or 48 h with one of the second stimuli. As a priming stimulus, we used 50 μ l either heat-killed *C. albicans* yeast or hyphae at two concentrations (1x10³/ml or 1x10⁴/ml), β -glucan (100 μ g/ml), mannan (100 μ g/ml), three *C. albicans* mutants and their corresponding reintegrants: heat-killed *och1* Δ null mutant, *pmr1* Δ null mutant, *mnn4* Δ null mutant, *och1* Δ /*och1* Δ /*OCH1*, *pmr1* Δ /*pmr1* Δ /*PMR1* and *mnn4* Δ /*mnn4* Δ /*MNN4* revertant strains (1x10⁴/ml), as well as their

parental strain NGY152 (1×10^4 /ml). After 24 h, cells were subjected to a second stimulation with various stimuli, in a volume of 50 μ l. The second stimulus was one of the following: LPS (10 ng/ml), Pam3Cys4 (10 μ g/ml), heat killed *E. coli* (1×10^8 /ml), heat-killed *S. aureus* (1×10^7 /ml), or heat killed *B. fragilis* (1×10^7 /ml) in a final volume of 200 μ l. After 24 or 48 h, supernatants were collected and stored at -80°C until assayed. To determine whether our priming reagents were endotoxin free, we performed a series of experiments in the presence of polymyxin B. Prior to priming, the reagents were incubated for 3 h with polymyxin B (2 μ g/ml). To investigate whether priming could be blocked, we preincubated PBMCs for 1 h with dectin-1/CLEC7A Ab (dectin-1 antagonist; 10 μ g/ml) or with the isotype control (10 μ g/ml), Syk inhibitor (50 nM) or Raf-1 inhibitor (50 nM), before priming with β -glucan. After this, cells were treated with either RPMI or β -glucan (100 μ g/ml) and incubated for 24 h, followed by a second stimulation with LPS (10 ng/ml) for an additional 24 h. Cytokines were measured after the second stimulation.

Cytokine assay. The concentrations of TNF- α and IL-1 β (R&D Systems) and IL-6 and IL-10 (Sanquin, Amsterdam, The Netherlands) were measured in cell culture supernatants using ELISA, according to the manufacturer's instructions. Proinflammatory cytokines TNF- α , IL-1 β , and IL-6 were measured at 24 h after the second stimulation, whereas the anti-inflammatory cytokine IL-10 was measured at 48 h after the second stimulation.

Statistical analysis. Results from at least two sets of experiments with a minimum four volunteers were pooled and analyzed with SPSS. Data are given as mean \pm SEM. The paired Wilcoxon test was used to compare differences between the effect of a particular stimulus and the RPMI medium control. The level of significance was set at $p < 0.05$.

Results

C. albicans primes the cytokine responses of cells restimulated with TLR ligands and colonizing bacteria

To investigate the effect of *Candida* on the cytokine responses to colonizing bacteria, PBMCs from healthy donors were first exposed to low doses of *Candida*. After 24 h, cells were restimulated with common colonizing bacteria of the skin or gut or with pure components of the bacterial or fungal cell wall (Fig. 1A). Exposure to either *Candida* yeast or hyphae enhanced the production of TNF- α , IL-6, and IL-1 β (Fig. 1B, 1C) of PBMCs incubated with a second bacterial stimulus, such as *E. coli*, *S. aureus*, or *B. fragilis*. This priming effect was also observed when bacterial cell wall components, such as LPS, found in the outer membrane of Gram-negative bacteria, and the lipopeptide analogs of the N-terminal part of bacterial lipoprotein, Pam3Cys4, were used as a second stimulus. Notably, the increased production of proinflammatory cytokines did not correlate with a decrease in the anti-inflammatory cytokine IL-10 (Fig. 1B, 1C). A 10-fold lower concentration of *Candida* yeast or hyphae was also able to prime but to a lesser extent (~ 2 -fold less).

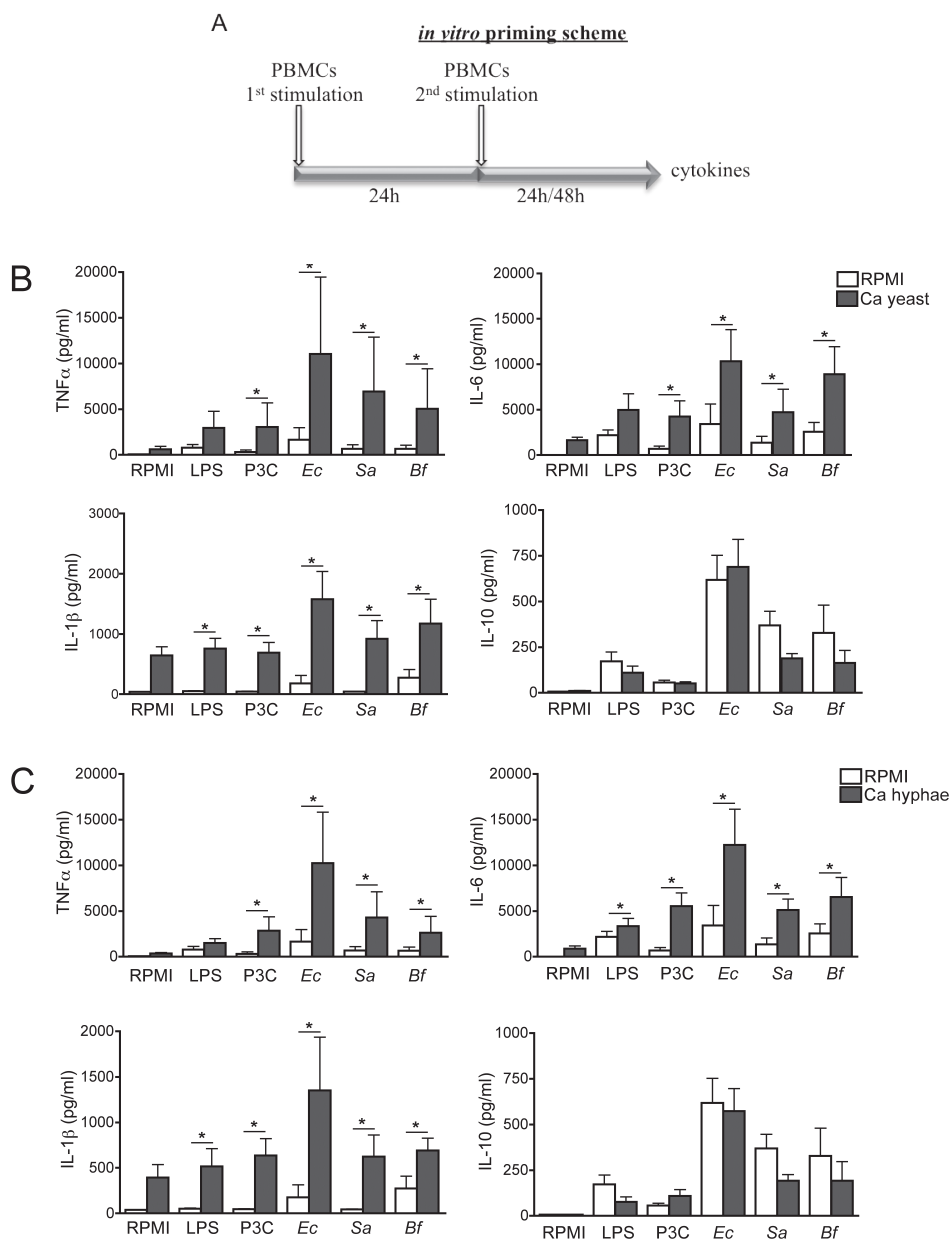


Fig. 1: *C. albicans* primes the cytokine responses of cells restimulated with TLR ligands and colonizing bacteria. (A) *In vitro* priming scheme. PBMCs were incubated with *Candida* (priming stimulus) for 24 h at 37°C. Thereafter, bacteria found in the human microbiota, as well as their cell wall pure components, were added (second stimuli) for an additional 24 or 48 h. PBMCs were exposed to RPMI or heat-killed *C. albicans* yeast (B) or hyphae (C) (1×10^4 cells/ml). After 24 h, PBMCs were exposed to several pure ligands or heat-killed bacteria. Cytokines were measured in supernatants 24 h (TNF- α , IL-1 β , and IL-6) and 48 h (IL-10) after the second stimulation (n=6; three independent experiments). Bars indicate mean \pm SEM. *p, 0.05 versus RPMI-primed cells, Wilcoxon nonparametric test for two related samples. Bf, *B. fragilis*; Ec, *E. coli*; P3C, Pam3Cys4; Sa, *S. aureus*.

Priming is not due to endotoxin contamination

Prior to priming, all stimuli (RPMI, β -glucan, and mannan) were incubated with cell culture medium (control) or with polymyxin B for 3 h at 37°C. Thereafter, PBMCs were primed for 24 h and then restimulated with LPS, Pam3Cys4 or cell culture medium only (RPMI) for an additional 24 h (Supplemental Fig. 1). Polymyxin B treatment of β -glucan did not affect its ability to prime PBMCs, excluding the effect of endotoxin contamination in the described effect. Mannan was still not able to prime, either with or without polymyxin B. In addition, β -glucan and polymyxin B priming was completely abrogated when PBMCs were restimulated with LPS; this was not the case for the same mix and restimulation with Pam3Cys4.

Mannans do not prime cytokine production by themselves but are essential for potentiation by *Candida*

The outer layer of the cell wall of *Candida* is enriched with mannoproteins, whereas the inner layer is composed of chitin and β -glucan (19). Mannosylated proteins and phospholipomannan of *Candida* are known to induce proinflammatory cytokines (20, 21). Therefore, we assessed the priming effect of pure mannans using the *in vitro* experimental setting depicted in Fig. 1A. We tested three purified preparations of *Candida* mannan as priming stimuli; none produced a priming effect compared with the nonprimed control (Fig. 2). Because mannans may potentiate priming by other cell wall components, we used a genetic complementary approach to investigate the role of specific mannosyl residues. The *Candida och1* null mutant has a defect in *N*-linked mannosylation (Fig. 3A), resulting in a 77% reduction of cytokine production in direct stimulations (19). In contrast, the *Candida mnn4* mutant, defective in tertiary branch mannosylphosphorylation (Fig. 3A) (17, 22), has no effect on the direct induction of cytokine production. *Candida pmr1* null mutant has a defect in phosphomannosylation, as well as in *O*- and *N*-linked glycosylation (18) (Fig. 3A). In our preincubation model, both *Candida och1* and *pmr1* null mutants had a partial defect in priming properties and could not potentiate the production of proinflammatory cytokines to the same extent as the parental strain (Fig. 3B, 3C). Of note, the priming effect

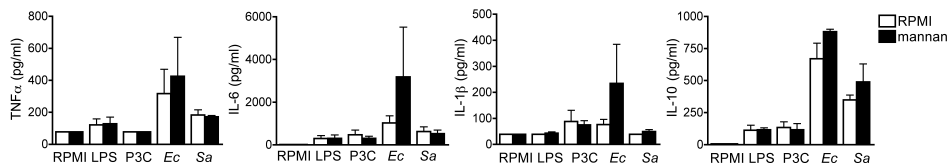


Fig. 2: By themselves, mannans do not prime cytokine production, but they are essential for potentiation by *C. albicans*. PBMCs were exposed to either RPMI or *Candida* mannan isolated from *Candida* hyphae grown in serum at 37°C at a concentration of 100 μ g/ml. After 24 h, PBMCs were restimulated with several pure ligands or heat-killed bacteria. Cytokines were measured in supernatants 24 h (TNF- α , IL-1 β , and IL-6) and 48 h (IL-10) after the second incubation. Three types of *Candida* mannan were tested, but none of them induced priming compared with the nonprimed control ($n=5$; two independent experiments). No statistical differences were detected using the Wilcoxon nonparametric test for two related samples. Ec, *E. coli*; P3C, Pam3Cys4; Sa, *S. aureus*.

was restored when cells were exposed to the reconstituted strains, *och1Δ/och1Δ/OCH1* and *pmr1Δ/pmr1Δ/PMR1* (Fig. 3B, 3C). The *mn4* null mutant was still able to prime cytokine production to the same extent as the parental strain *Candida* NGY152, and the same was valid for the reconstituted strain *mn4Δ/mn4Δ/MNN4* (Fig. 3D). Altogether, these results show that, although pure mannans do not prime for the production of proinflammatory cytokines, O- and, especially, N-linked mannosyl proteins at the surface of *Candida* are nevertheless required for the proper priming.

β-glucan primes the response to components of human microbiota through dectin-1 and Raf-1

Although β-glucan is a component of the inner layer of the *Candida* cell wall, it is a potent inducer of cytokines through its interaction with dectin-1 (23). Therefore, we assessed the effect of β-glucan in the priming model. Incubation of PBMCs with purified β-glucan from *Candida* was able to exert a priming effect similar to that obtained with *Candida* whole organism (Fig. 4A). The effect was most prominent for TNF-α and IL-1β and less so for IL-6. The β-glucan priming led to a decreased amount of the anti-inflammatory cytokine IL-10 induced by the secondary stimuli (Fig. 4A). When we blocked the dectin-1 receptor with an anti-dectin-1 Ab before priming with β-glucan, the priming effect was reduced considerably, whereas the isotype control had no effect (Fig. 4B). We completed the analysis using PBMCs from two sisters with congenital dectin-1 deficiency (24). In these experiments β-glucan priming was completely abolished, highlighting the importance of the dectin-1 receptor in mediating the β-glucan-dependent priming of cytokine production upon exposure to TLR ligands and colonizing bacteria (Fig. 4C). Dectin-1 signaling is primarily mediated by Syk/CARD9 (25). The inhibition of Syk kinase before β-glucan stimulation did not affect the priming and subsequent enhancement of TNF-α release upon LPS restimulation (Fig. 4D). However, inhibiting the noncanonical serine-threonine Raf-1 kinase (26) significantly decreased the priming effect (Fig. 4E). Altogether, these results show that β-glucan, a major component of the cell wall of *Candida*, exerts a strong priming effect toward restimulation with TLR ligands and colonizing bacteria and that this effect is mediated through the β-glucan receptor dectin-1 and the noncanonical Raf-1 kinase pathway.

Because live *Candida* cell walls consist of β-glucan masked by a layer of mannosylated proteins, it is possible that the simultaneous priming of PBMCs with β-glucan and mannan induces the production of proinflammatory cytokines to the same extent as does whole *Candida*. However, the priming effect of the two major *Candida* cell wall components, β-glucan and mannan, was not different from that of β-glucan alone (Fig. 5). These results underscore our observation that the priming of *Candida* is due to β-glucan and not to mannan.

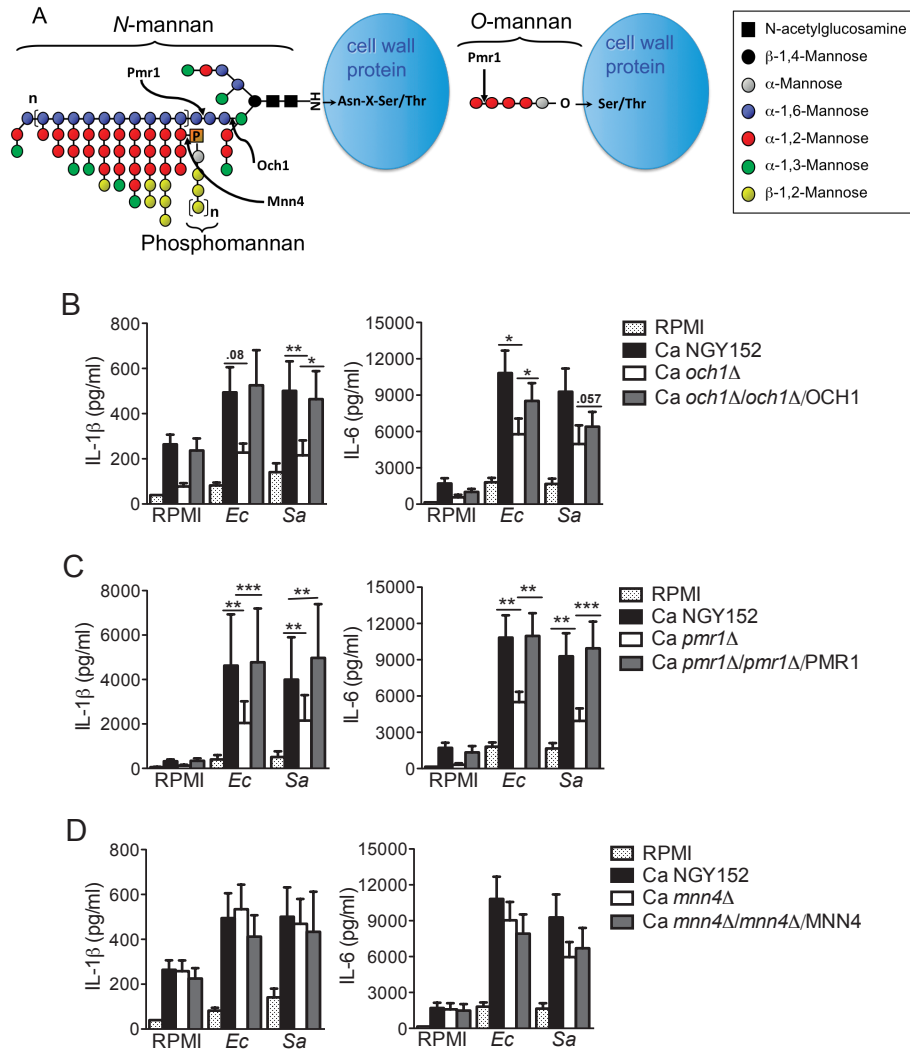


Fig. 3: Mannosylation of fungal cell wall proteins is involved in the potentiation of cytokine production. (A) Schematic diagram representing the activity of Och1, Pmr1, and Mnn4 enzymes on *C. albicans* cell wall glycosylation. For N-mannosylation, the Och1 mannosyltransferase mediates the addition of the first α-1,6-mannose to the core N-mannan structure. Two α-1,6-mannose residues form the backbone onto which α-1,2-mannose- and α-1,3-mannose-linked residues are subsequently attached by different enzymes to form the N-linked outer chain. Phosphomannan is attached to this outer chain via a phosphodiester bond, which requires Mnn4. Pmr1 is not a glycosyltransferase and does not add any mannans directly but exerts its effects on both N-mannosylation and O-mannosylation. The arrows represent where truncations in N-mannosylation and O-mannosylation occur. (B–D) PBMCs were exposed to NGY152, *och1*Δ, *pmr1*Δ, *mnn4*Δ, *och1*Δ/*och1*Δ/OCH1, *pmr1*Δ/*pmr1*Δ/PMR1, or *mnn4*Δ/*mnn4*Δ/MNN4 (10^4 cells/ml). After 24 h, PBMCs were restimulated with heat-killed bacteria. Cytokines (IL-1β and IL-6) were measured in supernatants 24 h after the second incubation (n=10; four independent experiments). Bars indicate mean ± SEM. All *Candida* strains induced a significant increase in proinflammatory cytokine production compared with nonprimed (RPMI) cells, whereas *och1*Δ and *pmr1*Δ null mutants resulted in significantly less priming of cytokine production compared with the parental strain or their corresponding reconstituted strains. *p, 0.05, **p, 0.01, ***p, 0.001, Wilcoxon nonparametric test for two related samples. *Ec*, *E. coli*; *Sa*, *S. aureus*.

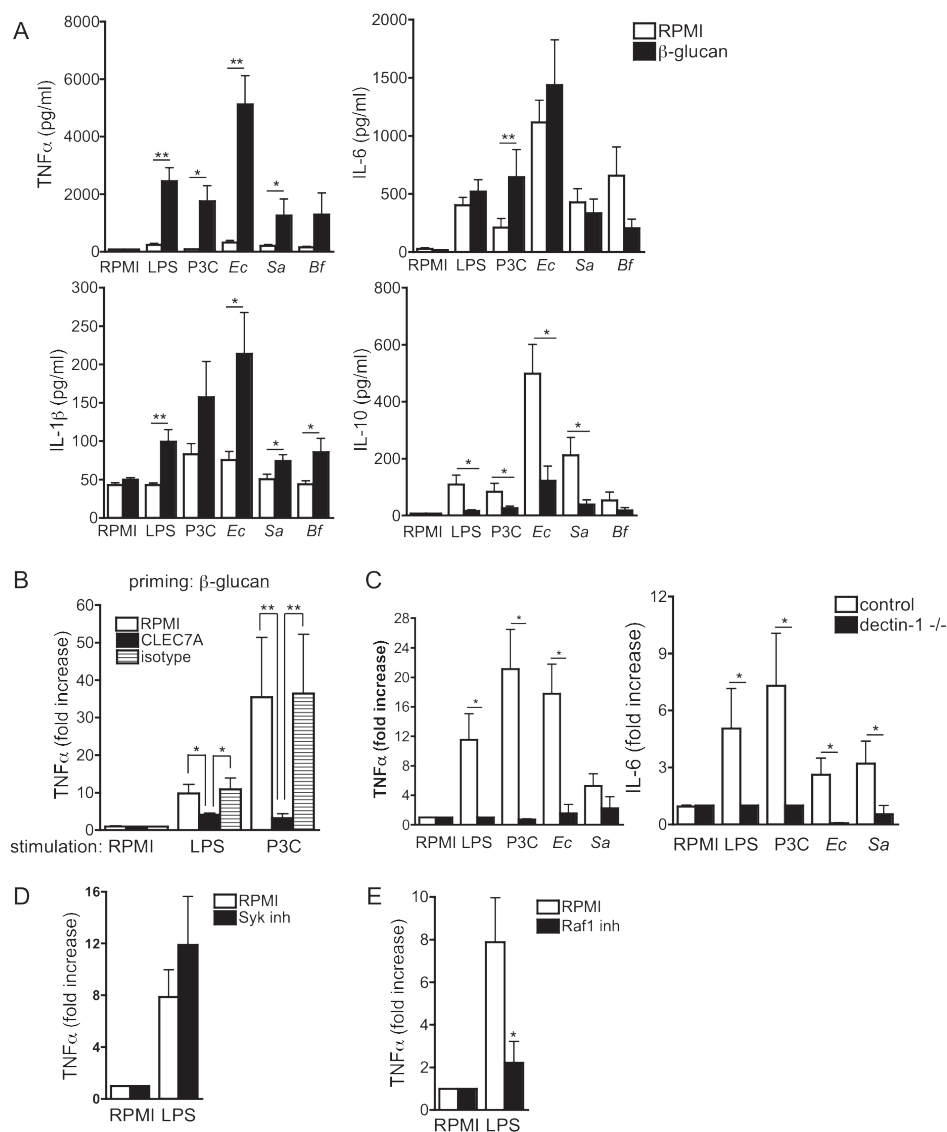


Fig. 4: β -glucan primes the response to components of human microbiota. (A) PBMCs were incubated with RPMI or β -glucan (100 μ g/ml). After 24 h, PBMCs were reincubated with pure ligands or heat-killed bacteria. Cytokines were measured in supernatants 24 h (TNF- α , IL-1 β , and IL-6) and 48 h (IL-10) after the second stimulation ($n=11$; five independent experiments). (C) PBMCs from 16 healthy volunteers and 2 dectin-1-deficient sisters (24) were incubated with RPMI and β -glucan (100 μ g/ml) for 24 h, followed by a second stimulation with either TLR ligands or whole bacteria for an additional 24 h. PBMCs were incubated with RPMI, CLEC7A Ab, and the isotype control (10 μ g/ml) (B), Syk inhibitor (1 mM) (D), or Raf-1 inhibitor (50 nM) (E). After 1 h of incubation, cells were treated either with RPMI or β -glucan (100 μ g/ml) and, after an additional 24 h, restimulated with LPS. For (B), $n=7$; three independent experiments; for (D) and (E), $n=10$; five independent experiments. Bars indicate mean \pm SEM. * p , 0.05, ** p , 0.01 versus RPMI-stimulated cells, Wilcoxon nonparametric test for two related samples. Bf, *B. fragilis*; Ec, *E. coli*; P3C, Pam3Cys4; Sa, *S. aureus*.

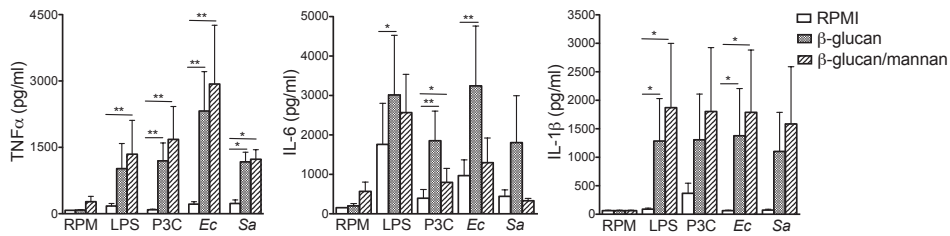


Fig. 5: Priming with a combination of β-glucans and mannans. PBMCs were incubated simultaneously with β-glucan and mannan (100 μg/ml); after 24 h, cells were stimulated either with TLR ligands or with whole bacteria for an additional 24 h (n=7; three independent experiments). Bars indicate mean ± SEM. *p, 0.05, **p, 0.01 versus RPMI-stimulated cells, Wilcoxon nonparametric test for two related samples. Ec, *E. coli*; P3C, Pam3Cys4; Sa, *S. aureus*.

Discussion

In the current study, we analyzed the capacity of the human commensal yeast *C. albicans* to modulate the cytokine response of human primary cells to bacteria that usually colonize the skin and mucosal surfaces. *Candida*, either yeast or hyphae, primes the cells for enhanced proinflammatory cytokine production when they are exposed to a secondary bacterial stimulus. This priming activity is dependent on carbohydrate components of the cell wall of *Candida*, with an important role for the recognition of β-glucan by the dectin-1/Raf-1 pathway.

An important aspect is which molecular pattern of *Candida* is responsible for this increased immune activity. Combining purified cell wall components and genetic approaches, we established that fungal mannan itself does not prime PBMCs for the production of cytokines. Surprisingly, however, neither the och1 nor the pmr1 null *Candida* mutant was able to prime the production of proinflammatory cytokines at the same extent as was wild-type *Candida* or parental strain NGY152, whereas the use of a reconstituted strain entirely restored the priming effect. Thus, the presence of O- and N-linked mannosyl proteins at the *Candida* cell surface is required for proper priming, supposedly by aiding cell priming by an additional cell wall component. Of note, although, under aqueous conditions, long chains of purified β-glucan form a right-handed triple helical complex that is required for the proper binding to pattern recognition receptors (27–29), purified mannan might not have the required structure to be recognized by its receptor, offering an alternative explanation for the lack of priming induced by pure mannans. Different biological effects of mannans were reported, including induction of proinflammatory cytokines (19, 30) and of IL-10 (31). It was shown that when *Candida* mannoprotein was administered to mice before or during immunization with viable *Candida*, it downregulated mannan-specific delayed hypersensitivity (32), whereas mannoproteins confer protection to candidiasis and might contain epitopes involved in this protective response (32). In this study, we show that mannans incorporated in *Candida* cell wall have adjuvant activity for the priming effect exerted by this yeast.

In search of the main component of the *Candida* cell wall that induces priming, we demonstrate that β-glucan can mimic the priming effect obtained upon *Candida* preincubation. This effect of β-glucan is in line with previous studies showing that β-glucan

is an immunostimulant and, moreover, displays antitumor activity (33, 34). The receptor mediating the priming effects of β -glucans is dectin-1, as shown by experiments with cells genetically deficient for this receptor. Downstream signaling occurs via two pathways: the canonical Syk/CARD9 and the noncanonical serine-threonine kinase Raf-1 (25, 26, 35). We found that inhibition of Syk kinase, prior to exposure to β -glucan, did not influence the priming activity of β -glucan. In contrast, the incubation of PBMCs with a Raf-1 inhibitor prior to first stimulation significantly reduced the effect. This observation distinguishes the direct effect of dectin-1 stimulation (Syk dependent) from the priming effect mediated by dectin-1 (Raf-1 dependent). Indeed, a previous study showed a dual intracellular signaling upon dectin-1 activation, in which the Raf-1 pathway integrates with the Syk pathway at the level of NF- κ B (35). Because priming is induced by purified TLR ligands, which are important components of bacteria, we tend to conclude that the priming induced by *Candida* and β -glucans acts on TLR-dependent-signaling pathways.

Candida is a common commensal of the intestinal tract, and it was reported to increase the severity of intestinal inflammation in murine models of colitis (10, 11). The β -glucan receptor dectin-1 is involved in this effect, and polymorphisms in this receptor have been associated with the severity of ulcerative colitis (11) but not with the susceptibility to ulcerative colitis or Crohn's disease (36). Little is known about the mechanisms through which *Candida* may influence autoinflammatory diseases, such as Crohn's disease. Although simultaneous stimulation of cells with *Candida* derived polysaccharides and TLR ligands is known to be synergistic for cytokine induction, it is not known whether prestimulation of immune cells with *Candida* can prime or tolerize (as in the case of TLR stimulation with LPS or Pam3Cys4) the cells for subsequent stimulation with colonizing bacteria. In the current study, we demonstrate that preincubation of human primary cells with *Candida* primes them for the production of cytokines, and this amplification of inflammation may represent one mechanism for the increased severity of colitis induced by *Candida*. The priming effect found in this study may be relevant for these observations. Whether this effect of *Candida* is also relevant for inflammatory bowel disease in humans is not clear. However, because Abs toward *C. albicans* were shown to be associated specifically with Crohn's disease, one may speculate that *Candida* plays a role in this disease (37). It is attractive to hypothesize that this has to do with the priming effect described in this article.

Chronic stimulation of host immune cells by microorganisms may result in an enhanced functional state and, hence, increased resistance to infection or in immunosuppression (or tolerance). The latter may imply protection against tissue damage. Understanding the interplay between these two strategies may allow us to define how fungi adapt to the human immune system. It was proposed that *Candida* may either promote tolerance (9) or amplify inflammation and contribute to disease activity in Crohn's disease (11). In the current study, *Candida* did not induce tolerance, but it promoted the proinflammatory response of human primary cells to colonizing bacteria. Such priming may be related to the epigenetic reprogramming of *Candida* described in monocytes (38). Thus, the fungal microbiota may actively contribute to the state of host defense at mucosal sites. When leukocytes at the mucosal

sites previously primed by *Candida* encounter potentially pathogenic colonizing bacteria, the enhanced state of the host response may potentiate the defense (e.g., by recruitment and activation of effective immune cells and by induction of diffusion in mucosal sites).

In conclusion, we demonstrate that chronic exposure of primary human immune cells to *C. albicans* primes them for subsequent stimulation with TLR ligands and colonizing microorganisms, a mechanism that could explain the effects of this fungus on inflammatory bowel diseases. By identifying dectin-1/Raf-1 as one of the pathways responsible for these effects, we provide a potential therapeutic target in autoinflammatory diseases, such as Crohn's disease. Further studies are warranted to assess this hypothesis.

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Acknowledgments

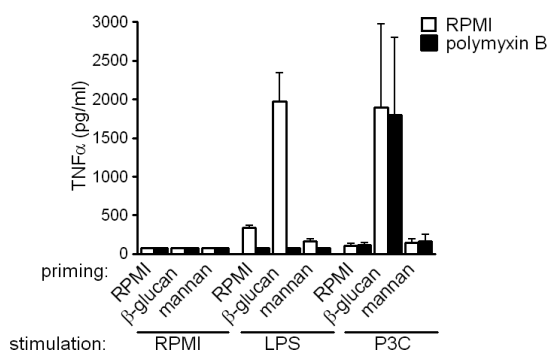
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References

- 1 Forssten SD, Sindelar CW, Ouwehand AC. Probiotics from an industrial perspective. *Anaerobe*. 2011 Dec;17(6):410-3.
- 2 Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol*. 2001 Oct;108(4):516-20.
- 3 O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep*. 2006 Jul;7(7):688-93.
- 4 Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol*. 2008 Oct;6(10):776-88.
- 5 Man SM, Kaakoush NO, Mitchell HM. The role of bacteria and pattern-recognition receptors in Crohn's disease. *Nat Rev Gastroenterol Hepatol*. 2011 Mar;8(3):152-68.
- 6 Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science*. 2012 Jun 8;336(6086):1268-73.
- 7 Odds FC. *Candida and candidosis*. Baltimore: University Park Press; 1979.
- 8 Calderone RA. *Candida and candidiasis*. Washington, D.C.: ASM Press; 2002.
- 9 Bonifazi P ZT, D'Angelo C, De Luca A, Moretti S, Bozza S, Perruccio K, Iannitti RG, Giovannini G, Volpi C, Fallarino F, Puccetti P, Romani L. Balancing inflammation and tolerance in vivo through dendritic cells by the commensal *Candida albicans*. *Mucosal Immunol* 2009;Jul;2(4):362-74.
- 10 Poulain D, Sendid B, Standaert-Vitse A, Fradin C, Jouault T, Jawhara S, et al. Yeasts: neglected pathogens. *Dig Dis*. 2009;27 Suppl 1:104-10.
- 11 Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. 2012 Jun 8;336(6086):1314-7.
- 12 Lowman DW, Ensley HE, Greene RR, Knagge KJ, Williams DL, Kruppa MD. Mannan structural complexity is decreased when *Candida albicans* is cultivated in blood or serum at physiological temperature. *Carbohydr Res*. 2011 Dec 13;346(17):2752-9.
- 13 Muller A, Rice PJ, Ensley HE, Coogan PS, Kalbfleish JH, Kelley JL, et al. Receptor binding and internalization of a water-soluble (1->3)-beta-D-glucan biologic response modifier in two monocyte/macrophage cell lines. *J Immunol*. 1996 May 1;156(9):3418-25.
- 14 Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol*. 2000 Jul 15;165(2):618-22.
- 15 Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics*. 1993 Jul;134(3):717-28.
- 16 Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, et al. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans*. *J Biol Chem*. 2006 Jan 6;281(1):90-8.
- 17 Hobson RP, Munro CA, Bates S, MacCallum DM, Cutler JE, Heinsbroek SE, et al. Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. *J Biol Chem*. 2004 Sep 17;279(38):39628-35.
- 18 Bates S, MacCallum DM, Bertram G, Munro CA, Hughes HB, Buurman ET, et al. *Candida albicans* Pmr1p, a secretory pathway P-type Ca²⁺/Mn²⁺-ATPase, is required for glycosylation and virulence. *J Biol Chem*. 2005 Jun 17;280(24):23408-15.
- 19 Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G, et al. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J Clin Invest*. 2006 Jun;116(6):1642-50.
- 20 Vecchiarelli A, Puliti M, Torosantucci A, Cassone A, Bistoni F. In vitro production of tumor necrosis factor by murine splenic macrophages stimulated with mannoprotein constituents of *Candida albicans* cell wall. *Cell Immunol*. 1991 Apr 15;134(1):65-76.
- 21 Ibata-Ombetta S, Idziorek T, Trinel PA, Poulain D, Jouault T. *Candida albicans* phospholipomannan promotes survival of phagocytosed yeasts through modulation of bad phosphorylation and macrophage apoptosis. *J Biol Chem*. 2003 Apr 11;278(15):13086-93.
- 22 Hazen KC, Singleton DR, Masuoka J. Influence of outer region mannosylphosphorylation on N-glycan formation by *Candida albicans*: normal acid-stable N-glycan formation requires acid-labile mannosylphosphate addition. *Glycobiology*. 2007 Oct;17(10):1052-60.
- 23 Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, et al. Immune recognition of

- Candida albicans beta-glucan by dectin-1. *J Infect Dis.* 2007 Nov 15;196(10):1565-71.
- 24 Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spruel AB, Venselaar H, et al. Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med.* 2009 Oct 29;361(18):1760-7.
- 25 Drummond RA, Saijo S, Iwakura Y, Brown GD. The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. *Eur J Immunol.* 2011 Feb;41(2):276-81.
- 26 Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity.* 2007 May;26(5):605-16.
- 27 Mueller A, Raptis J, Rice PJ, Kalbfleisch JH, Stout RD, Ensley HE, et al. The influence of glucan polymer structure and solution conformation on binding to (1->3)-beta-D-glucan receptors in a human monocyte-like cell line. *Glycobiology.* 2000 Apr;10(4):339-46.
- 28 Okobira T, Miyoshi K, Uezu K, Sakurai K, Shinkai S. Molecular dynamics studies of side chain effect on the beta-1,3-D-glucan triple helix in aqueous solution. *Biomacromolecules.* 2008 Mar;9(3):783-8.
- 29 Mishima Y, Quintin J, Aimanianda V, Kellenberger C, Coste F, Clavaud C, et al. The N-terminal domain of Drosophila Gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors. *J Biol Chem.* 2009 Oct 16;284(42):28687-97.
- 30 van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJ, Cheng SC, Joosten I, et al. The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe.* 2009 Apr 23;5(4):329-40.
- 31 Romani L. Immunity to fungal infections. *Nat Rev Immunol.* 2004 Jan;4(1):1-23.
- 32 Garner RE, Domer JE. Lack of effect of *Candida albicans* mannan on development of protective immune responses in experimental murine candidiasis. *Infect Immun.* 1994 Feb;62(2):738-41.
- 33 Nakao I, Uchino H, Orita K, Kaido I, Kimura T, Goto Y, et al. [Clinical evaluation of schizophyllan (SPG) in advanced gastric cancer--a randomized comparative study by an envelope method]. *Gan To Kagaku Ryoho.* 1983 Apr;10(4 Pt 2):1146-59.
- 34 Vetvicka V. Glucan-immunostimulant, adjuvant, potential drug. *World J Clin Oncol.* 2011 Feb 10;2(2):115-9.
- 35 Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC, et al. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat Immunol.* 2009 Feb;10(2):203-13.
- 36 de Vries HS, Plantinga TS, van Krieken JH, Stienstra R, van Bodegraven AA, Festen EA, et al. Genetic association analysis of the functional c.714T>G polymorphism and mucosal expression of dectin-1 in inflammatory bowel disease. *PLoS One.* 2009;4(11):e7818.
- 37 Sendid B, Dotan N, Nseir S, Savaux C, Vandewalle P, Standaert A, et al. Antibodies against glucan, chitin, and *Saccharomyces cerevisiae* mannan as new biomarkers of *Candida albicans* infection that complement tests based on *C. albicans* mannan. *Clin Vaccine Immunol.* 2008 Dec;15(12):1868-77.
- 38 Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe.* 2012 Aug 16;12(2):223-32.

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Supplementary Figure 1: β -glucan and mannan purity. Prior to priming, all stimuli (RPMI, β -glucan and mannan) were incubated with cell culture medium (control) or polymyxin B (2 μ g/ml) for 3h, at 37°C. Thereafter, PBMCs were primed for 24 h and then restimulated with LPS or Pam3Cys4 (P3C) for an additional 24 h.

3

The role of Dectin-2 for host defense against disseminated candidiasis

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Jos W.M. van der Meer, Frank van de Veerdonk, and Mihai G. Netea

Submitted for Publication.

Abstract

Despite the fact that *Candida albicans* is an important human fungal pathogen and Dectin-2 is a major pattern recognition receptor for fungi, our knowledge regarding the role of Dectin-2 for the host defense against disseminated candidiasis is limited. Dectin-2 deficient (Dectin-2^{-/-}) mice were more susceptible to systemic candidiasis, and the susceptibility was mirrored by an elevated fungal load in the kidneys that correlated with the presence of large inflammatory foci. Phagocytosis of *Candida* by peritoneal macrophages lacking the Dectin-2 receptor was moderately decreased, while production of most of the macrophage-derived cytokines from Dectin-2^{-/-} mice with systemic candidiasis was decreased. No striking differences among several *Candida* mutants defective in mannans could be detected between naïve wild-type and Dectin-2^{-/-} mice, apart from the β -mannan-deficient *bmt1* Δ /*bmt2* Δ /*bmt5* Δ triple mutant, suggesting that β -mannan may partially mask α -mannan detection, which is the major fungal structure recognized by Dectin-2. Deciphering the mechanisms responsible for host defense against the majority of *C. albicans* strains represents an important step in understanding the pathophysiology of systemic candidiasis, which might lead to the development of novel immunotherapeutic strategies.

Key words: Dectin-2, *Candida albicans*, systemic infection

Introduction

Although *Candida albicans* is a regular commensal of the skin, mucosal and gut flora, in susceptible individuals it represents the main cause of vaginal, mucocutaneous and systemic candidiasis (1). Moreover, disseminated candidiasis has a very high mortality rate in immunocompromised patients with transplantation, chemotherapy, or extensive ICU stay (2). To maintain *C. albicans* in its commensal state, the human host defense system should be able to recognize the yeast and activate the immune responses to suppress the uncontrolled outgrowth of the microorganism. This recognition is mediated by pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) of the pathogens with subsequent induction of the activation of host defense. Among the four PRR classes, C-type lectin receptors (CLRs) are the most important family of receptors for the recognition of *Candida spp.*, mainly because the fungal cell wall structure is composed predominantly of polysaccharides such as α - and β -mannans (components of mannoproteins), β -glucans and chitin (3).

Dectin-2 is a C-type lectin receptor present mostly on dendritic cells, macrophages and neutrophils that recognizes α -mannan (4, 5), affording protection against systemic infection with *C. albicans* by inducing Th-17 immune responses (5). It signals through CARD9, resulting in subsequent NF- κ B activation (6). Dectin-2 forms heterodimers with Dectin-3, leading to proinflammatory responses during *C. albicans* infection (7). In addition to *C. albicans*, Dectin-2 also recognizes *C. glabrata*, and mice lacking Dectin-2 were less able to secrete Th1 and Th17 cytokines, to produce ROS and to activate pathways involved in phagocytosis during activation with this pathogen (8). Furthermore, Dectin-2 present on alveolar macrophages is involved in triggering airway inflammation mediated by the house dust mite (9), while on dendritic cells it primes the Th2 responses to this microorganism (10).

Despite these reports on the role of Dectin-2 for the recognition of fungal pathogens, only limited information is available regarding the role of Dectin-2 for triggering the innate immune responses (such as inflammation, neutrophil/macrophages phagocytosis and killing, etc) during disseminated candidiasis. In this study, we investigate the role of Dectin-2 as a pattern recognition receptor and an essential trigger of host defense in a murine model of systemic candidiasis. Additionally, we assessed whether mannans are the main fungal PAMP recognized by Dectin-2, by testing a broad panel of *Candida* cell wall mutants.

Materials and Methods

Generation of Dectin-2^{-/-} mice. Dectin-2^{-/-} mice were generated by Ozgene Pty Ltd, as described previously (11). Eight- to twelve-week-old female Dectin-2^{-/-} (*Clec4e*^{-/-}) mice on a C57BL/6J background were obtained from a breeding colony at the Central Animal Laboratory, Radboud University Medical Centre and used for the experiments when they were 20-25g. Age-matched C57BL/6J female mice were obtained from Charles River Wiga (Sulzfeld, Germany). The animals were fed standard Laboratory Chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experiments were repeated at least

twice with a minimum of four animals per time point. All experiments were approved by the Ethics Committee on Animal Experiments of Radboud University, Nijmegen.

Candida strains, culture media, and growth conditions. *C. albicans* ATCC MYA-3573 (UC 820), a strain described in detail elsewhere (12), was used in all experiments. *Candida* was grown and maintained on Sabouraud dextrose (SD) plates. For inoculum preparation, a single colony was grown in SD broth at 29°C for 24 h, with shaking. Cells were washed twice in sterile phosphate-buffered saline (PBS) and counted using a hemocytometer. Cell density was adjusted with PBS to the desired inoculum level. *Candida* yeast was heat-killed for 30 min at 95°C. To generate hyphae, yeast cells were inoculated and grown overnight at 37°C in culture medium adjusted to pH 6.4 with hydrochloric acid. Hyphae were killed by exposure to 95°C for 30 minutes and resuspended in culture medium to a hyphal inoculum size that originated from 1×10^8 CFU/ml.

The *OCH1* and *PMR1* genes were disrupted to generate strains with severe truncations in N- and O-linked mannan that were compared to controls strains in *och1Δ/och1Δ/OCH1* and *pmr1Δ/pmr1Δ/PMR1* in which a wild type copy of the disrupted gene was reintroduced as described (13-15). The phosphomannan-deficient *mn4Δ* serotype B null mutant, the parental and its revertant were constructed as described (16). The *bmt1Δ* (17), *bmt2Δ*, *bmt5Δ* (18) and *bmt1Δ/bmt2Δ/bmt5Δ* (Courjol *et al.*, unpublished data, (19)), β-mannosyl transferase mutants were constructed as previously described.

***C. albicans* infection model and fungal burden.** Wild-type and Dectin-2^{-/-} mice were injected via the lateral tail vein with *Candida* (2.5×10^5 CFU per mouse) in a 100 μL volume of sterile pyrogen-free PBS. Mice were monitored daily. For survival studies, groups of 10 mice were followed-up for a period of 28 days. For immunological and histological studies, a non-lethal experimental model of disseminated candidiasis was used, in which animals were injected with 1×10^5 CFU per mouse via the lateral tail vein. This lower dose of *Candida* was used in order to avoid a bias induced by differential mortality at various time points (20). Subgroups of 4 animals were sacrificed on day 3, 7, 14 or 28 days after infection. Tissues were collected and processed for fungal burden and cytokine analysis. To assess the tissue outgrowth of *Candida*, the liver and kidneys were removed aseptically, weighed, and homogenized in sterile PBS in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on SD agar plates, as described elsewhere (21). The colony-forming units were counted after 24 h of incubation at 29°C and expressed as log colony-forming units per gram of tissue.

Histopathology. Kidney samples from wild-type and infected mice were kept in formaldehyde until processed. Sections were dehydrated with xylene, rehydrated through a graded series of ethanol solutions, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) using conventional staining methods. All individual segments were evaluated for the presence and intensity of inflammation, as well as for the presence of fungi. Tissue sections were analyzed with a VisionTek™ digital microscope (Sakura), using VisionTek Live software.

In-vitro cytokine production. Peritoneal macrophages were isolated from mice by injecting 5 ml of ice-cold sterile PBS (pH 7.4) into the peritoneal cavity. After centrifugation and

washing, cells were resuspended in RPMI-1640 culture medium containing 1 mM pyruvate, 2 mM L-glutamine and 50 mg/L gentamicin. Cells were counted using a Z1 Coulter Particle Counter (Beckman Coulter, Woerden, The Netherlands), adjusted to 1×10^6 cells/ml and cultured in 96-well round-bottom microtiter plates (Costar, Corning, The Netherlands) at 1×10^5 cells/well, in a final volume of 200 μ l. After 24 hours of incubation with different stimuli at 37°C and 5% CO₂, the plates were centrifuged at 1400 x g for 8 min, and the supernatants were collected and stored at -80°C until cytokine assays were performed.

Spleen cells were isolated by gently passing spleens through a sterile 200 μ m filter chamber. After washing with sterile PBS and centrifugation at 4°C (1200 rpm for 5 min), cells were resuspended in 2 ml RPMI-1640 in the presence of 20% FCS, counted and concentrations were adjusted to 1×10^7 cells/ml. Cells were cultured in 24-wells plates (Greiner, Alphen a/d Rijn, The Netherlands) at 5×10^6 cells/well, and different stimuli were added in a final volume of 1000 μ l. Supernatants were collected at two different time points (depending on the cytokine) as following: after 48 h of incubation at 37°C and 5% CO₂, 500 μ L supernatant per well were collected and stored at -80°C until cytokine assays were performed; thereafter, the plates were further incubated at 37°C and 5% CO₂ for 3 more days. Ultimately, the plates were centrifuged at 1400 x g for 8 min, and the remaining supernatants were collected and stored at -80°C until cytokine assays were performed. Concentrations of mouse TNF- α , IL-1 α and IL-1 β were determined by specific radioimmunoassay (RIA). Mouse IL-6, KC, IL-17, IFN- γ , IL-22 and IL-10 concentrations were measured by commercial ELISA kits (BioSource, Camarillo, CA), according to the instructions of the manufacturer.

Leukocyte recruitment. Wild-type and Dectin-2^{-/-} mice were injected with 100 μ l of 1×10^5 heat-killed *Candida* per mouse, intraperitoneally. After 4 hours cells were recruited from the peritoneum and 50 μ l of cell suspension (1×10^6 cells per ml) was centrifuged in a cytospin at 500 rpm for 10 minutes. The cells were stained with May-Grünwald Giemsa, observed and counted under the microscope.

Phagocytosis and killing of *C. albicans*. Peritoneal macrophages were recruited and phagocytosis and killing assays were performed as described elsewhere (8, 22).

Ethics statement. All experiments in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Dutch law on Animal experiments, and FELASA regulations. The protocol was approved by the Ethics Committee on Animal Experiments of the Radboud University Medical Centre. All efforts were made to minimize suffering of the animals.

Statistical analysis. Differences in phagocytosis, intracellular killing, and cytokine production were analyzed using the Mann-Whitney U-test. Survival data were analyzed using the Kaplan-Meier log rank test. Differences were considered significant at $P < 0.05$. The experiments were performed at least twice. At least 4 mice per group per time point were used for the outgrowth, phagocytosis, killing, and cytokine synthesis. 10 mice/group entered the survival experiment, which was repeated two more times. Data are presented as means \pm SEM.

Results

Dectin-2^{-/-} mice are more susceptible to systemic infection with *C. albicans*

A lethal dose of *Candida* was intravenously injected in wild-type and Dectin-2^{-/-} mice, which were thereafter monitored for 28 days. Dectin-2^{-/-} mice had significantly lower survival than wild-type mice (Fig. 1). On day 3, 7 and 14 post infection, organs of infected mice were isolated and CFU were counted. Interestingly, slightly fewer colonies were isolated from the livers of Dectin-2^{-/-} mice than of the wild-type mice at the early time points; however, both groups of mice were able to reduce the fungal burden in the liver (Fig. 2A). On the other hand, an increase of 100-fold *Candida* colonies were found in the kidneys of Dectin-2^{-/-} mice compared with the wild-type mice at late time points post-infection (Fig. 2B), which most likely is the cause of the increased mortality in the deficient mice.

This observation was further underlined by histological examination of the kidneys of Dectin-2^{-/-} mice, which developed inflammatory foci that were significantly larger than in wild-type mice (Fig. 2C-E, 2H). The foci consist mainly of lymphocytes with sporadic neutrophils (Fig. 2F and G), suggesting chronic inflammation, similar to systemic infection caused by *C. glabrata* (8). These results indicate that Dectin-2 is very important in the clearance of *Candida* predominantly from the kidneys, the target organ of disseminated candidiasis in mice.

The impact of Dectin-2 deficiency on cytokine production capacity

To obtain a greater understanding of immunological responses during systemic candidiasis in Dectin-2^{-/-} mice, we analyzed the cytokine production capacity during the course of infection with *C. albicans*. Interestingly, on day 3 and day 7 the production of proinflammatory cytokines (TNF- α , IL-6, IL-1 α and IL-1 β) by macrophages of Dectin-2^{-/-} mice were significantly lower than in wild-type mice (Fig. 3A), which may represent an important cause of decreased resistance to the pathogen. On the contrary, on days 14 and 28 the production of

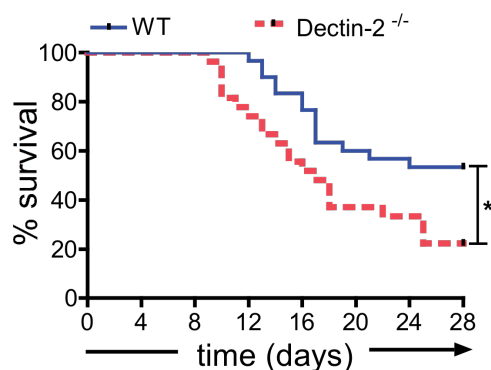


Figure 1: Dectin-2^{-/-} mice are more susceptible to *C. albicans* systemic infection. Survival curves of C57BL/6J wild-type (n = 20, continuous line) and Dectin-2^{-/-} (n = 19, interrupted line) mice after intravenous injection with live *C. albicans* UC820 (2.5×10^5 cells/mouse). *p = 0.012 by logrank test.

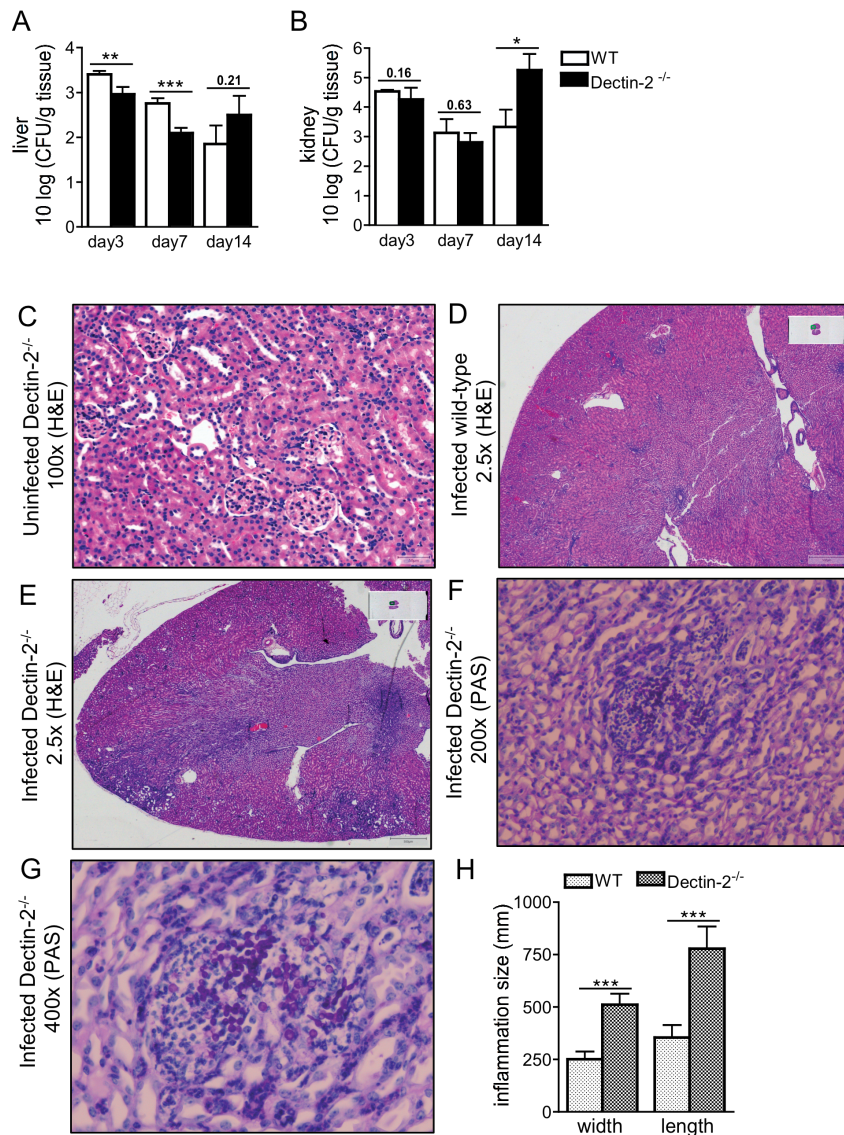


Figure 2: *Candida* outgrowth in the liver and kidneys of mice during systemic infection. *Candida* CFUs from liver (A) and kidneys (B) from wild-type and Dectin-2^{-/-} mice at day 3, 7 and 14 post-infection. Wild-type and Dectin-2^{-/-} mice received 1×10^5 CFU live *C. albicans* per animal intravenously. Results are presented as means \pm SEM (n=9-12 mice per group from 3 independent experiments). Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Histopathological assessment of kidneys on day 14 in uninfected Dectin-2^{-/-} (C), infected wild-type (D), infected Dectin-2^{-/-} (E) (hematoxylin and eosin stained sections) and infected Dectin-2^{-/-} (F and G) mice (periodic acid–Schiff stained sections). Representative localized lesions and *C. albicans* colonies are shown in the kidneys of Dectin-2^{-/-} mice (D and E). The dimension of inflammatory foci is represented in Figure H. A minimum of 20 foci were measured in at least 7 different kidneys. Results are means \pm SEM (n=4 mice per group) from 2 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as ***, $P < 0.001$.

proinflammatory cytokines in the knockout mice recovered, but this is most likely due to the increase of fungal burdens at these late time points (Fig. 3A).

We next analyzed the splenocyte-derived cytokines from mice with systemic candidiasis. Surprisingly, the production of TNF- α , IL-17, and IL-22 were moderately elevated from the splenocytes of knockout mice, particularly at day 3 post-infection (Fig. 3B), while mostly leveling up during the late course of infection (Fig. 3B). Interestingly, the production of the anti-inflammatory cytokine IL-10 was significantly decreased at day 28 after infection (Fig. 3B).

3

The role of mannans for *C. albicans* recognition by Dectin-2

In order to assess whether mannans are the ligands for Dectin-2, as previously suggested in the literature (4, 5), we screened several α - and β -mannan mutants for their ability to stimulate cytokine production by peritoneal macrophages and splenocytes from Dectin-2^{-/-} mice and wild-type controls. The macrophages from wild-type mice responded with normal production of TNF- α , IL-6, KC, IL-1 α and IL-1 β when stimulated with the majority of the *C. albicans* mutants (Fig. 4A). Interestingly, the triple mutant lacking β -mannan, *bmt1 Δ /bmt2 Δ /bmt5 Δ* , induced significantly higher amounts of IL-6 and KC than its parental strain *C. albicans* CAI4-CIP10, implying that α -mannan ligands are better accessible for recognition by cellular receptors on macrophages, as the triple mutant only lacks β -mannosides at the terminal end of α -mannosides, and phosphomannose on mannan and on phospholipomannan. Therefore, the lack of β -mannosides at the terminal end of α -mannosides suggests that α -mannans are still present on *Candida* triple mutant and, furthermore, are more exposed on *Candida* cells as they are no more hidden by β -mannosides. *Candida* hyphae and *och1 Δ* mutant induced sizable amounts of IL-1 α and IL-1 β in macrophages (Fig. 4A). Likewise, all mutants induced TNF- α , IL-17 and IL-22 in splenocytes from wild-type mice, while IFN- γ and IL-10 production was low (Fig. 4B).

Peritoneal macrophages from naïve Dectin-2^{-/-} mice stimulated with *C. albicans* mutants produced lower amounts of cytokines than wild-type mice (Fig. 5A). Moreover, the triple mutant *bmt1 Δ /bmt2 Δ /bmt5 Δ* did not induce more cytokines (in particular IL-6 and KC) than other mutants (Fig. 5A), suggesting that exposed α -mannan putative ligands interact with Dectin-2.

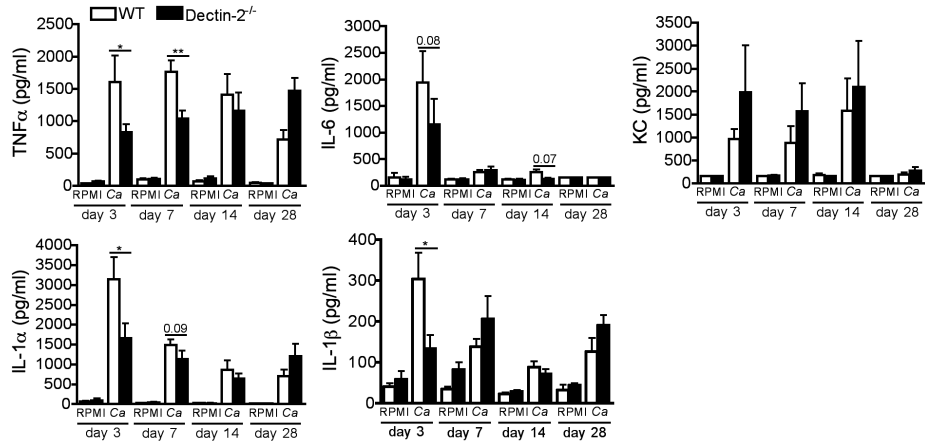
Decreased neutrophil recruitment at the site of infection in Dectin-2^{-/-} mice

Since neutrophils and monocytes are essential during fungal invasion, we investigated leukocyte recruitment after *Candida* injection into the peritoneal cavity of both Dectin-2^{-/-} and wild-type mice. The recruitment of neutrophils was clearly inhibited in knockout mice (Fig. 6A), while no differences in the recruitment of other leukocyte populations were observed. This observation, in combination with our finding of an increased level of the neutrophil chemokine KC in Dectin-2 knockout macrophages, suggests that Dectin-2 is important for the neutrophil response to KC.

Phagocytosis and killing of *Candida* by Dectin-2^{-/-} macrophages

Since a number of C-type lectin receptors are involved in the engulfment and phagocytosis of fungi, we investigated the phagocytic capacity of peritoneal macrophages from Dectin-2 knockout

A: macrophages



B: splenocytes

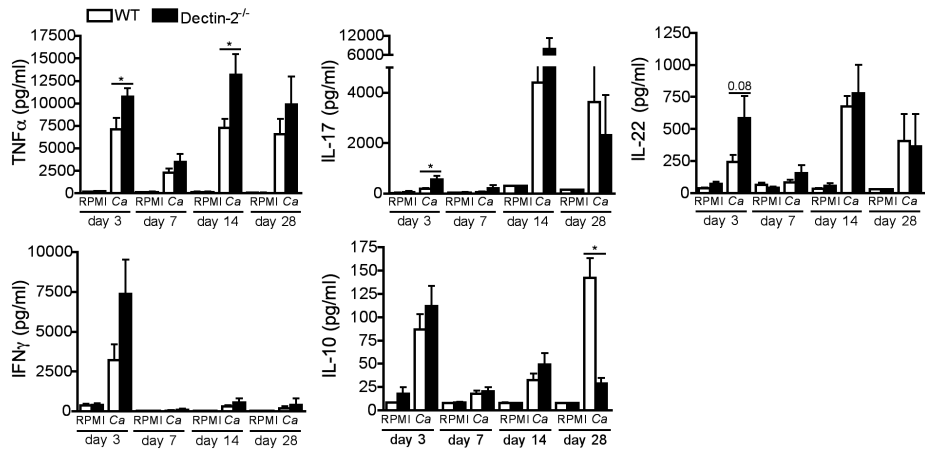


Figure 3: Cytokine profile during systemic infection with *C. albicans*. Wild-type or Dectin-2^{-/-} mice received 1*10⁵ CFU heat-killed *C. albicans* per mouse, intravenously. After 3, 7, 14 and 28 days, peritoneal macrophages (A) were restimulated *ex vivo* with heat-killed *C. albicans* hyphae (Ca) for 24 h. TNF-α, IL-6 and KC accumulation in the supernatants and IL-1α and IL-1β accumulation inside the cells was measured by ELISA. Results are means ± SEM (n=8 mice per group) from 2 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, P < 0.001; **, P < 0.01. Wild-type or Dectin-2^{-/-} mice received 1*10⁵ CFU live *C. albicans* per mouse, intravenously. After 3, 7, 14 and 28 days, splenocytes (B) were restimulated *ex vivo* with heat-killed *C. albicans* hyphae (Ca) for 48 h and 5 days. TNF-α, IL-10, IFNγ (48 h), IL-17 and IL-22 (5 days) accumulation in the supernatants was measured by ELISA. Results are means ± SEM (n=8 mice per group) from 2 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, P < 0.05.

Figure 2 displays four bar charts showing the levels of various cytokines (TNF α , IL-6, IL-1 α , and KC) in different bacterial strains. The y-axis represents the concentration in pg/ml. The x-axis lists the bacterial strains: RPMI, Ca v, Ca hy, NG v, NG hy, ocd1 Δ , ocd1 Δ ocd1 Δ , pmr1 Δ , pmr1 Δ pmr1 Δ , mmm4 Δ , mmm4 Δ mmm4 Δ , mmm4 Δ pmr1 Δ , mmm4 Δ pmr1 Δ mmm4 Δ , mmm4 Δ pmr1 Δ mmm4 Δ pmr1 Δ , bmt1 Δ , bmt1 Δ bmt1 Δ , bmt1 Δ bmt1 Δ bmt1 Δ , and bmt1 Δ bmt1 Δ bmt1 Δ bmt1 Δ . The charts show that the levels of these cytokines are significantly higher in the mmm4 Δ pmr1 Δ mmm4 Δ pmr1 Δ mmm4 Δ pmr1 Δ strain compared to the other strains.

[illegible]

◀ **Figure 4:** Stimulation of cells isolated from wild-type mice with *C. albicans* mutants. *C. albicans* mutants lacking O- and N-linked, α - and β -mannan were screened for their capacity to induce cytokines in wild-type peritoneal macrophages (A) and splenocytes (B) from naïve mice. Values represent means \pm SEM (n=4 mice per group, 2 independent experiments). Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Ca y: *C. albicans* yeast heat-killed; Ca hy: *C. albicans* hyphae heat-killed. The control strain NGY152, was derived from CAI-4, which is the parental Ura-auxotrophic strain of all mutants analysed, and was created by reintegration of a copy of *URA3* gene at the *RPS1* locus.

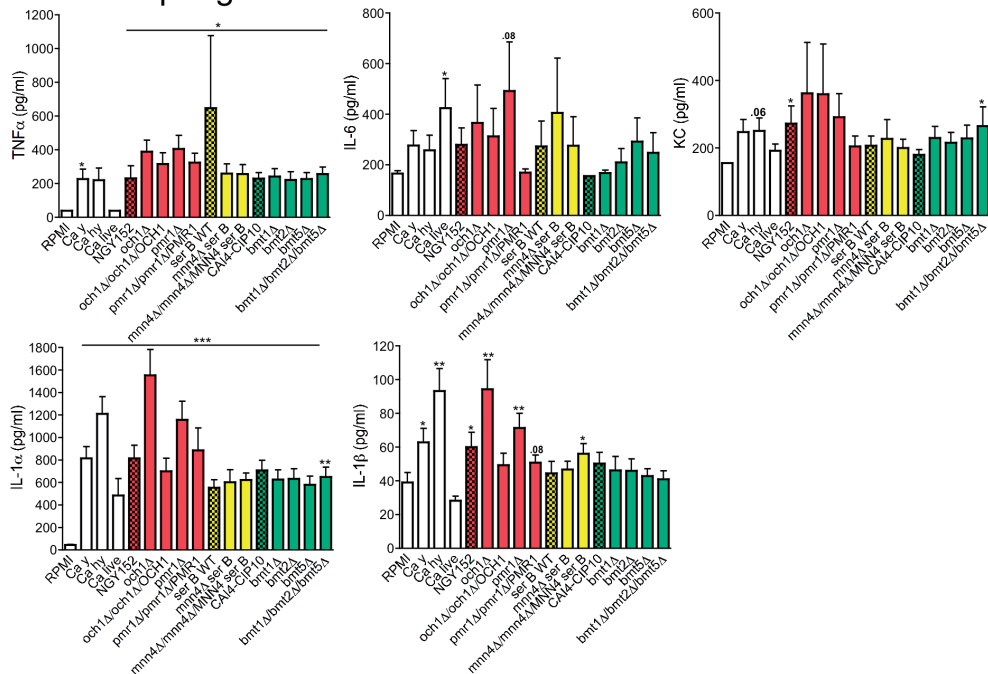
mice. We consistently found a slightly reduced phagocytosis of *Candida* by the macrophages from Dectin-2^{-/-} compared to the wild-type macrophages (Fig. 6B), suggesting a role of Dectin-2 in engulfment of the pathogen. The killing capacity, corrected for the decrease in phagocytosis, was not significantly decreased in macrophages of the knockout animals (Fig. 6B).

Discussion

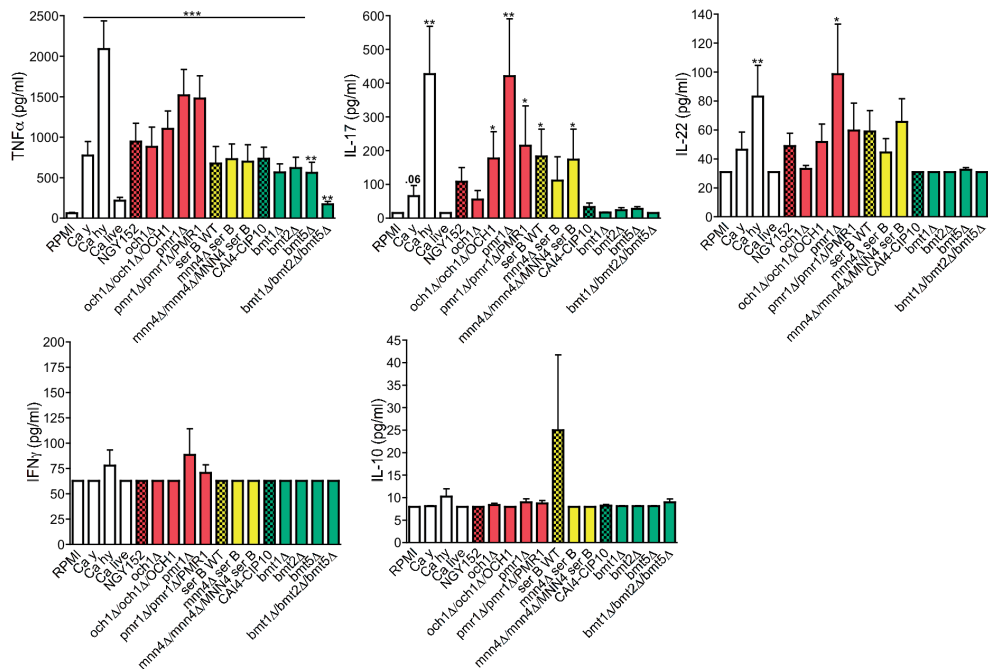
Our results demonstrate that mice lacking Dectin-2 are more susceptible to systemic *C. albicans* infection and we describe the altered host defense mechanisms that are responsible for this effect. The knockout mice exhibit diminished neutrophil recruitment into the peritoneal cavity, slightly decreased phagocytosis of *Candida* and larger inflammatory foci mostly devoid of neutrophils due to a higher fungal burden in the infected kidneys. We found that the macrophages production was lower for the majority of proinflammatory cytokines (with the exception of KC), which may represent the cause of these effects.

Our findings regarding an increased susceptibility of Dectin-2^{-/-} mice to *C. albicans* corroborate with those of Saijo *et al* (5), who demonstrated similar survival curves of Dectin-2 knockout mice challenged with *C. albicans*. In addition, these findings are in line with the increased susceptibility of Dectin-2^{-/-} mice to *C. glabrata* (8). Macrophage-derived proinflammatory cytokines such as TNF- α , IL-1, KC or IL-6 activate the recruitment, phagocytosis and killing of fungi, and mice deficient in these cytokines are more susceptible to systemic candidiasis (23, 24). Interestingly, with the exception of KC, significant defects in the production of these cytokines were observed in cells isolated from *Candida*-challenged Dectin-2^{-/-} mice in our study. Saijo *et al* previously reported a much more dramatic decrease in cytokine production compared to the data reported here, i.e. non detectable IL-6, TNF- α , IL-1 β and IL-10 after exposure of mononuclear phagocytes to *Candida* yeasts and very low production after exposure of Dectin-2^{-/-} cells to hyphae. Our data show more moderate effects, which is in line with the well-known redundancy in the various pattern recognition receptors responsible for the recognition of *Candida spp* (25). Even a greater disparity with the findings of Saijo *et al* (5) was found with regard to cytokine production by splenocytes. At day 14 of infection, a significant amount of IL-17 was produced by splenocytes of Dectin-2^{-/-} mice, demonstrating that the Th17 differentiation in these mice is functional. The increased production of cytokines by splenocytes of Dectin-2^{-/-} mice at certain time points after infection is most likely due to the higher antigen burden in the organs of the knockout mice. This is also discrepant with the findings of Saijo *et al.*, and the cause of these differences

A: macrophages from Dectin-2^{-/-} naïve mice



B: splenocytes from Dectin-2^{-/-} naïve mice



◀ **Figure 5:** Stimulation of cells isolated from Dectin-2^{-/-} mice with *C. albicans* mutants. *C. albicans* mutants lacking O- and N-linked, α- and β-mannan were screened for their capacity to induce cytokines in Dectin-2^{-/-} peritoneal macrophages (A) and splenocytes (B) from Dectin-2^{-/-} mice. Values represent means ± SEM (n=4 mice per group, 2 independent experiments). Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, P < 0.05; **, P < 0.01; ***, P < 0.001. Ca y: *Candida albicans* yeast heat-killed; Ca hy: *Candida albicans* hyphae heat-killed.

remains to be investigated. One likely explanation may be represented by differences between various *C. albicans* strains: such differences have been earlier reported to cause differential effects of various strains for the recognition of TLR4 (26, 27) and Dectin-1 (27).

The coordinated induction of proinflammatory cytokines is important for activating multiple host defense mechanisms against fungi, including leukocyte recruitment at the site of infection, phagocytosis and killing of the pathogen. The influx of neutrophils was decreased in the knockout mice, despite normal concentrations of the chemoattractant cytokine KC, pointing to a role of Dectin-2 in the chemotactic response of neutrophils. In addition, we observed that peritoneal macrophages lacking Dectin-2 were slightly less able to ingest the pathogen, while the killing of *Candida* was not affected. Phagocytosis is the main mechanism through which immune cells engulf and clear pathogens or cell debris. The latter observation of normal *Candida* killing by Dectin-2^{-/-} cells is remarkable, since we have previously shown that Dectin-2^{-/-} neutrophils produce less reactive oxygen species (ROS) upon stimulation (8), and ROS plays a significant role in *Candida* killing (28). Drewniak *et al* showed that human CARD9 deficiency resulted in selective defect in the host defense against invasive fungal infection caused by an impaired phagocyte killing (28); this suggest that CARD9-dependent C-type lectin receptors other than Dectin-2 are also important for *Candida* killing.

The increased susceptibility of Dectin-2^{-/-} mice to *C. albicans* strain UC820 is suggested to be due to their incapacity to eliminate the fungus from the kidneys, the target organ of

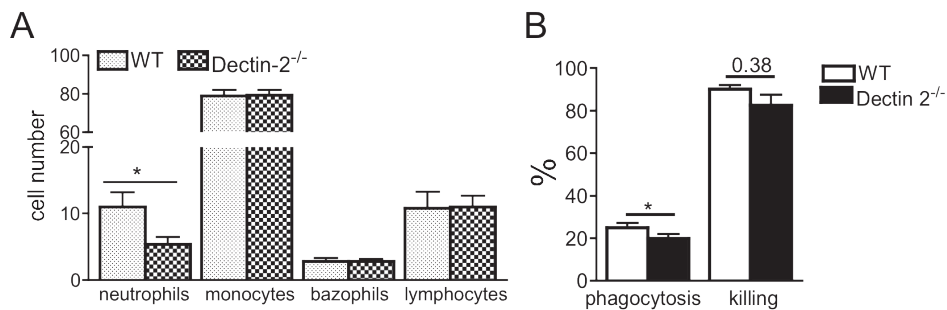


Figure 6: The role of Dectin-2 for leukocyte antifungal biological properties. Leukocyte recruitment in the peritoneum (A): wild-type and Dectin-2^{-/-} mice were injected intraperitoneally with 100 µl of 1x10⁵ heat-killed *C. albicans* per mouse. After 4 hours cells were recruited, stained with May-Grünwald Giemsa, and counted under the microscope. Phagocytosis and killing of *C. albicans* cells by murine peritoneal macrophages (B): the results are represented as percentage of phagocytosis (the percentage of fungal cells engulfed by macrophages present in the well) and percentage of killing (the percentage of killed fungal cells among the phagocytosed yeast). The *C. albicans*/macrophage ratio was 10:1. Values represent means ± SEM (n=5 mice per group) *, P < 0.05.

disseminated candidiasis (29). The fact that the animals were able to eradicate *Candida* from the liver but not kidney confirms previous data related to organ-specific *Candida* infection and immune responses mediated primarily by neutrophils and monocytes (30-32). The ultimate pathways responsible for the increased susceptibility of Dectin-2^{-/-} mice to systemic candidiasis is most likely due to the combination of defective cytokine production, reduced neutrophil recruitment and impaired phagocytosis of the fungus.

α-mannans have been previously suggested to be the main fungal PAMP recognized by Dectin-2 (5). Furthermore, Dectin-2 binds to the terminal mannose of N-linked glycan (4), and recognizes mycobacterial mannose-capped lipoarabinomannan (33), and O-linked mannanose-rich glycoproteins (α-1,2-linked mannose) from *Malassezia* (34). Considering these studies, we were surprised to observe that mutant *C. albicans* strains with defects in α-mannans induced largely intact cytokine amounts. Moreover, the mannan-defective strains induced less cytokines in macrophages from Dectin-2^{-/-} mice compared with the wild-type animals. In accord with this, *och1Δ* and *pmr1Δ*, induced lower Th-derived cytokines in splenocytes of wild-type and Dectin-2^{-/-} mice previously infected with *Candida*, compared to the other strains of the fungus. Finally, the triple mutant lacking β-mannan and therefore exposing α-mannan, *bmt1Δ/bmt2Δ/bmt5Δ*, induced significantly higher amounts of IL-6 and KC than its parental strain *C. albicans* CAI4-CIP10, confirming that, indeed, *C. albicans* α-mannan ligands are better accessible for recognition by cellular receptors on macrophages. Moreover, *bmt1Δ/bmt2Δ/bmt5Δ* did not induce more cytokines than other mutants in the Dectin-2^{-/-} cells, suggesting that these exposed α-mannan ligands interact with Dectin-2.

In conclusion, we demonstrate that Dectin-2 is an important component of the anti-*Candida* host immune responses. However, the extent of immune responses triggered by Dectin-2 during systemic candidiasis may vary depending not only on *Candida* species, but also on the strain of *Candida*. Deciphering the precise mechanisms responsible for host defense against the different *C. albicans* strains represents an important step in understanding of the pathophysiology of systemic candidiasis. Furthermore, revealing the exact Dectin-2 ligand(s) important for induction of antifungal host defense mechanisms could lead to the development of novel immunotherapeutic strategies and possible vaccine development.

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Conflicts of interest

The authors declare no conflict of interest.

References

- 1 Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. 2012 Jun 8;336(6086):1314-7.
- 2 Yapar N. Epidemiology and risk factors for invasive candidiasis. *Ther Clin Risk Manag*. 2014;10:95-105.
- 3 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol*. 2008 Jan;6(1):67-78.
- 4 McGreal EP, Rosas M, Brown GD, Zamze S, Wong SY, Gordon S, et al. The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology*. 2006 May;16(5):422-30.
- 5 Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity*. 2010 May 28;32(5):681-91.
- 6 Bi L, Gojestani S, Wu W, Hsu YM, Zhu J, Ariizumi K, et al. CARD9 mediates dectin-2-induced I κ B kinase ubiquitination leading to activation of NF- κ B in response to stimulation by the hyphal form of *Candida albicans*. *J Biol Chem*. 2010 Aug 20;285(34):25969-77.
- 7 Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, et al. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. *Immunity*. 2013 Aug 22;39(2):324-34.
- 8 Ifrim DC, Bain JM, Reid DM, Oosting M, Verschuere I, Gow NA, et al. Role of Dectin-2 for host defense against systemic infection with *Candida glabrata*. *Infect Immun*. 2014 Mar;82(3):1064-73.
- 9 Clarke DL, Davis NH, Campion CL, Foster ML, Heasman SC, Lewis AR, et al. Dectin-2 sensing of house dust mite is critical for the initiation of airway inflammation. *Mucosal Immunol*. 2014 May;7(3):558-67.
- 10 Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, et al. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J Exp Med*. 2011 Mar 14;208(3):593-604.
- 11 Zhang Z, Lutz B. Cre recombinase-mediated inversion using lox66 and lox71: method to introduce conditional point mutations into the CREB-binding protein. *Nucleic Acids Res*. 2002 Sep 1;30(17):e90.
- 12 Lehrer RI, Cline MJ. Interaction of *Candida albicans* with human leukocytes and serum. *J Bacteriol*. 1969 Jun;98(3):996-1004.
- 13 Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics*. 1993 Jul;134(3):717-28.
- 14 Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, et al. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans*. *J Biol Chem*. 2006 Jan 6;281(1):90-8.
- 15 Bates S, MacCallum DM, Bertram G, Munro CA, Hughes HB, Buurman ET, et al. *Candida albicans* Pmr1p, a secretory pathway P-type Ca²⁺/Mn²⁺-ATPase, is required for glycosylation and virulence. *J Biol Chem*. 2005 Jun 17;280(24):23408-15.
- 16 Hobson RP, Munro CA, Bates S, MacCallum DM, Cutler JE, Heinsbroek SE, et al. Loss of cell wall mannosylphosphate in *Candida albicans* does not influence macrophage recognition. *J Biol Chem*. 2004 Sep 17;279(38):39628-35.
- 17 Fabre E, Sfihi-Loualia G, Pourcelot M, Coddeville B, Krzewinski F, Bouckaert J, et al. Characterization of the recombinant *Candida albicans* beta-1,2-mannosyltransferase that initiates the beta-mannosylation of cell wall phosphopeptidomannan. *Biochem J*. 2014 Jan 15;457(2):347-60.
- 18 Mille C, Fradin C, Delplace F, Trinel PA, Masset A, Francois N, et al. Members 5 and 6 of the *Candida albicans* BMT family encode enzymes acting specifically on beta-mannosylation of the phospholipomannan cell-wall glycosphingolipid. *Glycobiology*. 2012 Oct;22(10):1332-42.
- 19 Wilson RB, Davis D, Enloe BM, Mitchell AP. A recyclable *Candida albicans* URA3 cassette for PCR product-directed gene disruptions. *Yeast*. 2000 Jan 15;16(1):65-70.
- 20 Netea MG, Vonk AG, van den Hoven M, Verschuere I, Joosten LA, van Krieken JH, et al. Differential role of IL-18 and IL-12 in the host defense against disseminated *Candida albicans* infection. *Eur J Immunol*. 2003 Dec;33(12):3409-17.
- 21 Kullberg BJ, van 't Wout JW, van Furth R. Role of granulocytes in increased host resistance to *Candida*

- albicans induced by recombinant interleukin-1. *Infect Immun*. 1990 Oct;58(10):3319-24.
- 22 Kullberg BJ, van 't Wout JW, Hoogstraten C, van Furth R. Recombinant interferon-gamma enhances resistance to acute disseminated *Candida albicans* infection in mice. *J Infect Dis*. 1993 Aug;168(2):436-43.
- 23 Basu S, Quilici C, Zhang HH, Grail D, Dunn AR. Mice lacking both G-CSF and IL-6 are more susceptible to *Candida albicans* infection: critical role of neutrophils in defense against *Candida albicans*. *Growth Factors*. 2008 Feb;26(1):23-34.
- 24 Gorjestani S, Darnay BG, Lin X. Tumor necrosis factor receptor-associated factor 6 (TRAF6) and TGFbeta-activated kinase 1 (TAK1) play essential roles in the C-type lectin receptor signaling in response to *Candida albicans* infection. *J Biol Chem*. 2012 Dec 28;287(53):44143-50.
- 25 Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol*. 2012 Feb;10(2):112-22.
- 26 Netea MG, Gow NA, Joosten LA, Verschuere I, van der Meer JW, Kullberg BJ. Variable recognition of *Candida albicans* strains by TLR4 and lectin recognition receptors. *Med Mycol*. 2010 Nov;48(7):897-903.
- 27 Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, et al. Differential adaptation of *Candida albicans* in vivo modulates immune recognition by dectin-1. *PLoS Pathog*. 2013;9(4):e1003315.
- 28 Drewniak A, Gazendam RP, Tool AT, van Houdt M, Jansen MH, van Hamme JL, et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood*. 2013 Mar 28;121(13):2385-92.
- 29 Spellberg B, Ibrahim AS, Edwards JE, Jr., Filler SG. Mice with disseminated candidiasis die of progressive sepsis. *J Infect Dis*. 2005 Jul 15;192(2):336-43.
- 30 Lionakis MS, Lim JK, Lee CC, Murphy PM. Organ-specific innate immune responses in a mouse model of invasive candidiasis. *J Innate Immun*. 2011;3(2):180-99.
- 31 Lionakis MS, Fischer BG, Lim JK, Swamydas M, Wan W, Richard Lee CC, et al. Chemokine receptor Ccr1 drives neutrophil-mediated kidney immunopathology and mortality in invasive candidiasis. *PLoS Pathog*. 2012;8(8):e1002865.
- 32 Ngo LY, Kasahara S, Kumasaka DK, Knoblaugh SE, Jhingran A, Hohl TM. Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. *J Infect Dis*. 2014 Jan 1;209(1):109-19.
- 33 Yonekawa A, Saijo S, Hoshino Y, Miyake Y, Ishikawa E, Suzukawa M, et al. Dectin-2 Is a Direct Receptor for Mannose-Capped Lipoarabinomannan of *Mycobacteria*. *Immunity*. 2014 Aug 27.
- 34 Ishikawa T, Itoh F, Yoshida S, Saijo S, Matsuzawa T, Gonoi T, et al. Identification of distinct ligands for the C-type lectin receptors Mincle and Dectin-2 in the pathogenic fungus *Malassezia*. *Cell Host Microbe*. 2013 Apr 17;13(4):477-88.

4

Role of Dectin-2 for Host Defense against Systemic Infection with *Candida glabrata*

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Abstract

Although *Candida glabrata* is an important pathogenic *Candida* species, relatively little is known about its innate immune recognition. Here, we explore the potential role of Dectin-2 for host defense against *C. glabrata*. Dectin-2-deficient (*Dectin-2*^{-/-}) mice were found to be more susceptible to *C. glabrata* infections, showing a defective fungal clearance in kidneys but not in the liver. The increased susceptibility to infection was accompanied by lower production of T helper 1 (Th1) and Th17-derived cytokines by splenocytes of *Dectin-2*^{-/-} mice, while macrophage-derived cytokines were less affected. These defects were associated with a moderate yet significant decrease in phagocytosis of the fungus by the *Dectin-2*^{-/-} macrophages and neutrophils. Neutrophils of *Dectin-2*^{-/-} mice also displayed lower production of reactive oxygen species (ROS) upon challenge with opsonized *C. glabrata* or *C. albicans*. This study suggests that Dectin-2 is important in host defense against *C. glabrata* and provides new insights into the host defense mechanisms against this important fungal pathogen.

Keywords: *Candida glabrata*, Dectin-2, Th-derived cytokines, ROS

Introduction

The opportunistic fungal pathogen *Candida glabrata* is the second most frequent cause of candidiasis after *Candida albicans*, accounting for approximately 15 to 25% of *Candida* infections (1, 2). Similar to other *Candida* species, *C. glabrata* resides as a commensal within the normal microbial flora of humans, but it may also cause serious infections in immunocompromised patients. In the last 3 decades, the number of infections due to non-*albicans* *Candida* species has increased significantly (3, 4). The current rise in the incidence of invasive *C. glabrata* infections is largely the result of the combinational increase in immunosuppressive infections and modern prolonged invasive medical interventions (5). Moreover, *C. glabrata* is of added concern due to the fact that a fraction of clinical isolates have been proven to be resistant to antifungal agents, such as azoles (6). The mortality rate associated with bloodstream infection with *C. glabrata* is approximately 50% in cancer patients and is even higher in bone marrow transplant patients (7, 8). However, compared to the literature on *C. albicans*, few studies have investigated the pathophysiology of *C. glabrata* infections, despite its high morbidity and mortality burden.

Pattern recognition of fungal components and activation of innate immunity are essential steps in the host defense against *Candida* species (9). The *Candida* cell wall is mainly composed of multiple layers of carbohydrates, such as mannan, β -glucan, and chitin, which are collectively recognized by C-type lectins, including macrophage mannose receptor (MR), SIGNR-1, Galectin-3, Mincle, Dectin-1, Dectin-2, and the Toll-like receptors. This recognition leads to activation of the host innate immune system. MR recognizes the N-linked mannans of fungal cell walls, SIGNR-1 recognizes branched α -mannans, and Galectin-3 recognizes β -(1,2)-mannans (9–11). It has recently been reported that Dectin-1 (*Clec7a*) and Dectin-2 (*Clec4n*) are specific receptors for β -glucans (12) and *C. albicans*-derived α -mannans (13), respectively. Dectin-2 is a C-type lectin receptor primarily expressed by macrophages, dendritic cells and neutrophils, with specificity for structures rich in mannose (14, 15). Dectin-2 uses the FcR γ chain to signal via Syk- and caspase recruitment domain family member 9 (Card9)-dependent pathways (16–18). It represents an important receptor for the generation of the Th17-like adaptive immune response, coordinating the Th1-like responses together with Dectin-1 (17). Human Dectin-2 has a mannose recognition EPN motif (15, 19), strengthening the likelihood that Dectin-2 plays an important role in antifungal immunity. Additionally, Saijo *et al.* have reported that Dectin-2 is the functional receptor for α -mannans and that Dectin-2^{-/-} mice are more susceptible to *C. albicans* (13). However, little is known regarding the specific role of Dectin-2 for the host defense against *C. glabrata*.

In this study, we have explored the contribution of Dectin-2 in the pathophysiology of murine *C. glabrata* systemic infection. We show that the burden of *C. glabrata* was significantly increased in kidneys of Dectin-2^{-/-} mice, which was correlated with a decreased phagocytosis and killing of the fungus by the innate immune macrophages (as shown by live-cell video microscopy phagocytosis assays) and neutrophils (as shown by the *ex vivo* phagocytosis and killing assays). Moreover, the adaptive T helper-derived cytokine responses were also considerably affected in Dectin-2^{-/-} mice. In conclusion, our data

demonstrate an important role of Dectin-2 for innate immune responses and its significant contribution to the adaptive immune responses against *C. glabrata*.

Materials and Methods

Generation of Dectin-2^{-/-} mice. Dectin-2^{-/-} mice were constructed by Ozgene Pty Ltd., Australia, as described previously (20). Briefly, a targeting vector was designed to introduce a conditional mutation into the mouse *Clec4n* gene (Ensembl version no. http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000023349;r=6:123229843-123247021). The strategy utilizes mutant *loxP* sites (*lox66* and *lox71*) to enable an inversion of the flanked sequence in response to the expression of Cre recombinase. The recombination event inactivated the gene by switching the position and direction of the transcriptional termination (STOP) signal with *Clec4n* exon 2. The *lox66* site was inserted upstream from *Clec4n* exon 2, and a neomycin selection cassette (PGK-Neo) was targeted into the intron between exon 2 and exon 3. The selection cassette was flanked with FLP recombination target (FRT) sites to enable removal by FlpE-mediated recombination. An inverted STOP element and a *lox71* site were placed downstream from the selection marker. The 5' and 3' homology arms of the vector were approximately 6.1 kb and 5.9 kb, respectively. The targeting vector was linearized with PmeI and electroporated into the C57BL/6 embryonal stem (ES) cell line Bruce4. Neomycin-resistant clones were selected and screened by Southern blotting to identify homologous recombinants. Correctly targeted clones were microinjected into BALB/cJ blastocysts, which were transferred into pseudopregnant CBB6F1 foster females. Chimeric mice were obtained and outcrossed to C57BL/6J females to generate ES cell-derived targeted mutant progeny.

Eight- to 12-week-old female Dectin-2^{-/-} (*Clec4n*^{-/-}) mice on a C57BL/6J background were obtained from a breeding colony at the Central Animal Laboratory, Radboud University Nijmegen Medical Centre. Age-matched C57BL/6J female mice were obtained from Charles River Wiga (Sulzfeld, Germany). All mice weighed between 20 and 25 g. The animals were fed standard laboratory chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. Most of the experiments were repeated at least twice with a minimum of five animals per time point. All experimentation conformed to the terms and conditions of the Ethics Committee on Animal Experiments of Radboud University, Nijmegen. Key experiments, such as for fungal outgrowth and phagocytosis and killing of *C. glabrata*, were repeated using Dectin-2^{-/-} mice generated independently, kindly provided by Y. Iwakura (13). These validation studies were performed at the animal facility of the University of Aberdeen. Live-cell video microscopy was conducted in Aberdeen; C57BL/6 and Dectin-2^{-/-} (provided by Y. Iwakura) mice were obtained from breeding colonies. All mice were used at 8 to 16 weeks of age. Animals were kept and handled in accordance with institutional guidelines.

***Candida* strains, culture media, and growth conditions.** *C. glabrata* CBS138 (ATCC 2001), a strain described elsewhere (21), was used in all experiments. *C. glabrata* was routinely grown and maintained on Sabouraud dextrose plates. For inoculum preparation, a single colony was grown in Sabouraud dextrose broth at 29°C for 24 h, with shaking. Cells were washed

twice in sterile phosphate-buffered saline (PBS) and counted using a hemocytometer. Cell density was adjusted with PBS to the desired inoculum level. *C. glabrata* was heat killed for 30 min at 95°C. *Candida albicans* ATCC MYA-3573 (UC 820) was grown overnight to generate yeast cells in Sabouraud dextrose broth at 29°C, with shaking. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in culture medium (RPMI 1640 Dutch modification). *C. albicans* yeast was heat killed for 30 min at 95°C. To generate hyphae, yeast cells were inoculated and grown overnight at 37°C in culture medium adjusted to pH 6.4 with hydrochloric acid. Hyphae were killed by exposure to 95°C for 30 min and resuspended in culture medium to a hyphal inoculum size that originated from 1×10^8 CFU/ml.

***C. glabrata* infection model and fungal burden.** A nonlethal experimental model of disseminated candidiasis was used, in which wild-type and Dectin-2^{-/-} mice were injected intravenously, via the lateral tail vein, with *C. glabrata* (1×10^7 CFU/mouse) in a 100 μ l volume of sterile pyrogen-free PBS. Mice were monitored daily. For survival studies, groups of 10 mice were followed-up for a period of 28 days. For immunological and histological studies, subgroups of 5 animals were killed on day 3, 7, or 14 postinfection. Tissues were collected and processed for fungal burden and cytokine analysis. To assess the tissue outgrowth of *C. glabrata* on these days, the liver and kidneys were removed aseptically, weighed, and homogenized in sterile PBS in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates, as described elsewhere (22). The CFU were counted after 24 h of incubation at 29°C and expressed as log CFU per gram of tissue.

Histopathology. Kidney samples from infected mice were kept in formalin until processed. Sections were dehydrated with xylene, rehydrated through a graded series of ethanol solutions, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) using conventional staining methods. All individual segments were evaluated for the presence and intensity of inflammation, as well as for the presence of fungi. Tissue sections were analyzed with a VisionTek digital microscope (Sakura), using VisionTek Live software.

***In vitro* cytokine production.** Peritoneal macrophages were isolated from mice by injecting 5 ml of ice-cold sterile PBS (pH 7.4) into the peritoneal cavity. After centrifugation and washing, cells were resuspended in RPMI 1640 culture medium containing 1 mM pyruvate, 2 mM L-glutamine, and 50 mg/liter gentamicin. Cells were counted using a Z1 Coulter particle counter (Beckman Coulter; Woerden, The Netherlands), adjusted to 1×10^6 cells/ml, and cultured in 96-well round-bottom microtiter plates (Costar, Corning, The Netherlands) at 1×10^5 cells/well in a final volume of 200 μ l. After 24 h of incubation with different stimuli at 37°C and 5% were centrifuged at $1,400 \times g$ for 8 min, and the supernatants were collected and stored at -80°C until cytokine assays were performed.

Spleen cells were isolated by gently passing spleens through a sterile 200 μ m filter chamber. After washing with sterile PBS and centrifugation at 4°C (1,200 rpm for 5 min), cells were resuspended in 2 ml RPMI 1640 in the presence of 20% fetal calf serum and counted, and concentrations were adjusted to 1×10^7 cells/ml. Cells were cultured in 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands) at 5×10^6 cells/well, and different stimuli were

added in a final volume of 1,000 μ l. Supernatants were collected at two different time points (depending on the cytokine) as follows. After 48 h of incubation at 37°C and 5% CO₂, 500 μ l of supernatant per well was collected and stored at -80°C until cytokine assays were performed; thereafter, the plates were further incubated at 37°C and 5% CO₂ for an additional 3 days. The plates were centrifuged at 1,400 x g for 8 min, and the remaining supernatants were collected and stored at -80°C until cytokine assays were performed. The concentrations of mouse tumor necrosis factor alpha (TNF- α), interleukin-1 α (IL-1 α), and IL-1 β were determined by specific radioimmunoassay (RIA). Mouse IL-6, KC, IL-17, gamma interferon (IFN- γ), IL-22, and IL-10 concentrations were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (Biosource, Camarillo, CA) according to the instructions of the manufacturer.

Phagocytosis and killing of *C. glabrata*. Peritoneal macrophages and neutrophils were recruited and phagocytosis and killing assays were performed according to a modification of a method described elsewhere (23). *C. glabrata*/macrophage or *C. glabrata*/neutrophil ratios of 10:1 were used in the phagocytosis and killing studies. Peritoneal macrophages or neutrophils from groups of 5 C57BL/6J (control) and 5 Dectin-2^{-/-} mice were elicited by an intraperitoneal (i.p.) injection of heat-killed *C. glabrata*. Cells were collected in separate sterile tubes either 3 days (macrophages) or 4 h (neutrophils) after *C. glabrata* infection by washing the peritoneal cavity with 5 ml of ice-cold PBS. Phagocytes were centrifuged (for 10 min at 2,250 x g), counted in a Bürker counting chamber, and resuspended in RPMI 1640 Dutch modification (with 20 mM HEPES and without L-glutamine; ICN Biomedicals) supplemented with 5% heat-inactivated fetal calf serum, 1% gentamicin, 1% L-glutamine, and 1% pyruvate. The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes. To create a monolayer of phagocytes, 5x10⁵ cells in 100 μ l of RPMI 1640 were dispensed into the wells of a 96-well flat-bottom plate (Costar) and incubated at 37°C and 5% CO₂. Macrophages were allowed to adhere for up to 2 h, but neutrophils for only 30 min before the monolayers were gently washed with culture medium to remove nonadherent cells. The percentage of adherence was calculated as follows: $(1 - [\text{number of nonadherent cells} / 5 \times 10^5]) \times 100$. Subsequently, the cells were incubated with 1x10⁴ CFU *C. glabrata*, which were opsonized for 30 min at 24°C in modified Eagle's medium (MEM; Gibco Life Technologies) that contained 2.5% fresh mouse serum (effector/target ratio, 40:1). After 15 min, supernatants were aspirated, and monolayers were gently washed with MEM to remove noningested *C. glabrata*. The supernatant and well washings that contained the non-ingested *Candida* were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytosed microorganisms was defined as follows: $[1 - (\text{number of uningested CFU} / \text{CFU at the start of incubation})] \times 100$.

The killing of *C. glabrata* by phagocytes was assessed in the same monolayers. After removal of the nonphagocytized *Candida*, 200 μ l of culture medium, consisting of Sabouraud in MEM (50%, vol/vol), was added to the monolayers. After 3 h of incubation at 37°C and 5% CO₂, the wells were gently detached with a cell scraper and washed with 200 μ l distilled H₂O to achieve lysis of phagocytes. This procedure was repeated 3 times, after which the pooled washes were adjusted to a final volume of 1 ml with distilled water. Microscopic examination of the culture plates showed that there was an almost complete removal of phagocytes. To quantify the viable

intracellular *Candida*, 10-fold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the phagocytes was determined as follows: $[1 - (\text{CFU after incubation} / \text{number of phagocytized CFU})] \times 100$. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability.

Live-cell video microscopy phagocytosis assays. Standard phagocytosis assays were performed as previously described (24). Briefly, 6×10^5 live *C. glabrata* yeasts were added to 2×10^5 macrophages in μ -Slide 8-well chambers (ibidi GmbH, Germany) immediately prior to imaging. Video microscopy experiments were performed using an Ultra View VoX live-cell imaging system (PerkinElmer) with the environmental control chamber set at 37°C. Images were captured at 1-min intervals for 3 h using an electron-multiplying charge-coupled device (EMCCD) camera. At least two independent experiments were conducted for each mouse strain, and two movies for each mouse were analyzed ($n=7$ wild-type mice, and $n=8$ Dectin-2 mice). Volocity 6.2.1 imaging analysis software was used to track macrophage migration at 1-min intervals for the first 60 min of the phagocytosis assay. The software enabled high-throughput analysis of macrophage migration, providing detailed information on the distances traveled and the directionality and velocity of hundreds of individual macrophages. The data were subsequently displayed in tracking diagrams and used to calculate the mean track velocity and track length of macrophages cultured with *C. glabrata*. The movies generated for migration analyses were also analyzed to determine phagocytic uptake at the 30-min, 60-min, and 180-min time points (25). For each mouse, 20 macrophages were analyzed for the percentage of uptake of live *C. glabrata* yeasts (calculated by determining the number of macrophages per 100 that had ingested at least one yeast). The phagocytic index was calculated by counting the total number of yeasts engulfed per hundred macrophages at each time point.

Reactive oxygen species (ROS) assay. The spontaneous and stimulus induced oxygen radical production levels of isolated neutrophils were evaluated using luminol-enhanced chemiluminescence and determined in an automated LB96V MicroLumat plus luminometer (EG & G Berthold, Bald Wilberg, Germany) as previously described (26). In this study, neutrophils (2×10^5 per well) were seeded into 96-well plates and incubated in medium containing either RPMI, phorbol 12-myristate 13-acetate (PMA; 5 $\mu\text{g/ml}$), live opsonized *C. albicans* (10^5 CFU/ml) or live opsonized *C. glabrata* (10^7 CFU/ml). Luminol was added to each well in order to start the chemiluminescence reaction. Each measurement was carried out in at least duplicate repetitions. Chemiluminescence was determined every 145 s at 37°C for 1 h. Luminescence was expressed as relative light units (RLU) per second. Data were analyzed with Winglow software (EG & Berthold).

Ethics statement. All experiments in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Dutch law on Animal Experiments, and FELASA regulations. The protocol was approved by the Ethics Committee on Animal Experiments of the Radboud University Nijmegen Medical Centre. All efforts were made to minimize suffering of the animals. All experiments performed in Aberdeen conformed to the terms

and conditions of United Kingdom Home Office licenses for research on animals and the guidelines of the University of Aberdeen ethical review committee.

Statistical analysis. Differences in phagocytosis, postphagocytic killing, and concentrations of cytokines were analyzed using the Mann-Whitney U test. Survival data were analyzed using the Kaplan-Meier log rank test. Differences were considered significant at a *P* value of 0.05. The majority of the experiments was performed at least twice, and at least 5 mice/group/time point were used for the outgrowth, phagocytosis, killing, ROS, and cytokine synthesis experiments. A total of 10 mice/group were used for survival experiments. Data are presented as means \pm standard errors of the means (SEM).

Results

Increased susceptibility to *C. glabrata* systemic infection in Dectin-2^{-/-} mice

To determine the role of Dectin-2 in the host defense against *C. glabrata*, we first compared the susceptibility to infection of Dectin-2^{-/-} and wild-type control mice. Following infection with *C. glabrata*, both groups of mice were able to effectively control and eradicate the fungus from the liver within 2 weeks (Fig. 1A). In contrast, while wild-type animals restrained the infection in their kidneys, significantly higher *C. glabrata* loads were observed in Dectin-2^{-/-} mice (Fig. 1B). Similar increases in the fungal burdens were observed in the kidneys of the Dectin-2^{-/-} mice provided by Y. Iwakura (Fig. 1C). Nonetheless, Dectin-2^{-/-} mice were less susceptible to *C. glabrata* than has been reported for *C. albicans*, with no mortality recorded in any of the mouse strains during the 28 days following intravenous infection (data not shown).

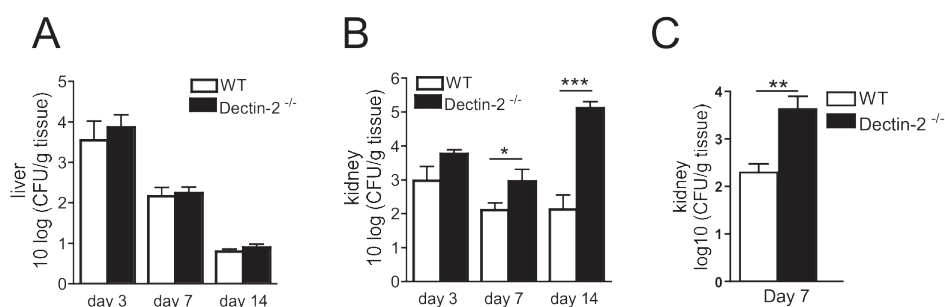


Fig. 1: Greater *C. glabrata* loads in the kidneys of mice during systemic infection. (A and B). Quantification of *Candida* colonies from liver (A) and kidneys (B) of wild-type and Dectin-2^{-/-} mice at day 3, 7 and 14 postinfection is shown. Wild-type and Dectin-2^{-/-} received 10⁷ CFU of *C. glabrata* per mouse intravenously. After 3, 7 and 14 days, organs were collected and serial dilutions were plated on Sabouraud solid media. Results are means \pm SEM (n=10-14 mice per group from 3 independent experiments). Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, *P* < 0.05; ***, *P* < 0.001. (C) Fungal burdens in the kidneys of wild-type and Dectin-2^{-/-} mice provided by Prof. Iwakura. Wild-type or Dectin-2^{-/-} mice received 10⁷ CFU of *C. glabrata* per mouse intravenously. After 7 days, kidneys were collected and serial dilutions were plated on Sabouraud agar media. Data are represented as means \pm SEM (n=12 mice per group from two independent experiments). Significance was determined with Mann-Whitney U-test, ** *P* < 0.01.

Compared with the results for noninfected mice (Fig. 2A), histopathological analysis identified zones with lymphocytic infiltration suggestive of chronic inflammation in both wild-type control (Fig. 2B) and Dectin-2^{-/-} (Fig. 2C) mice. Sporadic neutrophilic granulocytes could be detected (Fig. 2D and E). At day 7 and day 14 postinfection, the kidneys of Dectin-2^{-/-} mice displayed significantly increased numbers of inflammatory foci compared with the kidneys of wild-type mice. Interestingly, fungi were only partially associated with inflammation and were often present in the lumina of tubuli, not in the tissue itself (Fig. 2D and E). Therefore, the foci of chronic, lymphocytic inflammation were

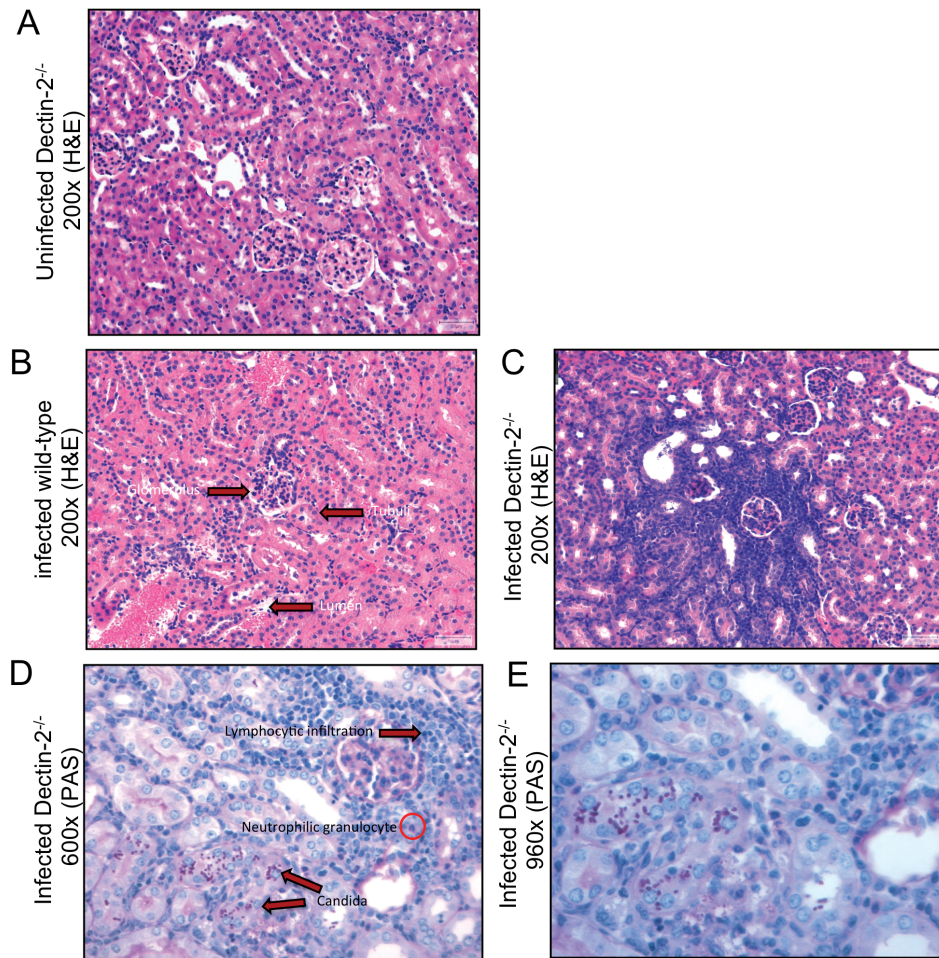


Fig. 2: Dectin-2 deficiency affects kidney histopathology during systemic infection with *C. glabrata*. Histopathological assessment of kidneys in uninfected Dectin-2^{-/-} (A), infected wild-type (B), infected Dectin-2^{-/-} (C) (hematoxylin and eosin stained sections) and infected Dectin-2^{-/-} (D and E) mice (periodic acid-Schiff stained sections). Representative localized lesions and *C. glabrata* colonies in the kidneys of Dectin-2^{-/-} mice are shown (C, D and E). All observations in infected kidneys were performed at day 14 after intravenous injection with *C. glabrata*.

not associated with the presence of fungi. In conclusion, Dectin-2 appears to be an essential pattern recognition receptor for the adequate control of *C. glabrata* renal infection.

Defective Th-associated cytokine production in Dectin-2^{-/-} mice

To further characterize the role of Dectin-2 for the recognition of *C. glabrata*, the *ex vivo* cytokine profile was assessed in peritoneal macrophages of naive wild-type and knockout mice. Interestingly, the innate response levels from uninfected Dectin-2^{-/-} macrophages were slightly higher than the response levels of the macrophages isolated from the wild-type animals (TNF- α , IL-6, IL-1 α , and IL-1 β) (Fig. 3A). Only the IL-6 and KC production levels from Dectin-2^{-/-} macrophages upon stimulation with *C. albicans* heat-killed hyphae were lower than those from wild-type macrophages, although the difference reached statistical significance only for KC (Fig. 3A). Regarding the T cell-derived cytokines (IL-10, IFN- γ , and IL-17) produced by the splenocytes of uninfected mice, no differences could be detected between the two groups of mice (Fig. 3B). The IL-17 production levels in the splenocytes from Dectin-2^{-/-} mice were highly variable.

In a subsequent set of experiments, we studied the *ex vivo* cytokine responses of peritoneal macrophages and splenocytes isolated from mice infected with *C. glabrata*. No differences were detectable between the two groups of mice in most of the macrophage-derived cytokines assayed (intracellular/extracellular IL-1 α and IL-1 β and extracellular production of IL-6 and KC; data not shown), with the exception of TNF- α at day 14 postinfection (Fig. 4A). Similar to the results for peritoneal macrophages, *ex vivo* stimulation of splenocytes from *C. glabrata*-infected mice resulted in significantly lower levels of TNF- α production by Dectin-2-deficient cells upon stimulation with either *C. glabrata* (Fig. 4B) or *C. albicans* (Fig. 4C), although dissimilarities were detectable earlier in the course of the infection. No significant differences could be detected in the amounts of the anti-inflammatory cytokine IL-10 produced by either strain. In addition to the responses of the innate cytokines, Th1 and Th17 responses at 7 days postinfection were also significantly reduced in Dectin-2^{-/-} splenocytes, as mirrored by the low production of IFN- γ and IL-17 (Fig. 4B). The levels of IL-22 were moderately reduced at day 14 postinfection (Fig. 4B). A similar pattern with more pronounced differences was observed when splenocytes from infected mice were restimulated with *C. albicans* yeast (Fig. 4C). As such, Dectin-2 deficiency results in decreased production of protective T helper-derived cytokines.

Dectin-2^{-/-} peritoneal macrophages are less efficient in the uptake and killing of *C. glabrata ex vivo*

The phagocytic clearance of microorganisms, including yeasts, by professional phagocytes is mediated by receptor activation that drives the engulfment and internalization of the pathogen. We therefore assessed the ability of Dectin-2^{-/-} peritoneal macrophages to recognize, phagocytose, and kill live opsonized *C. glabrata*. Using a classical *ex vivo* phagocytosis and killing assay (23), wild-type peritoneal macrophages were found to be slightly more efficient in

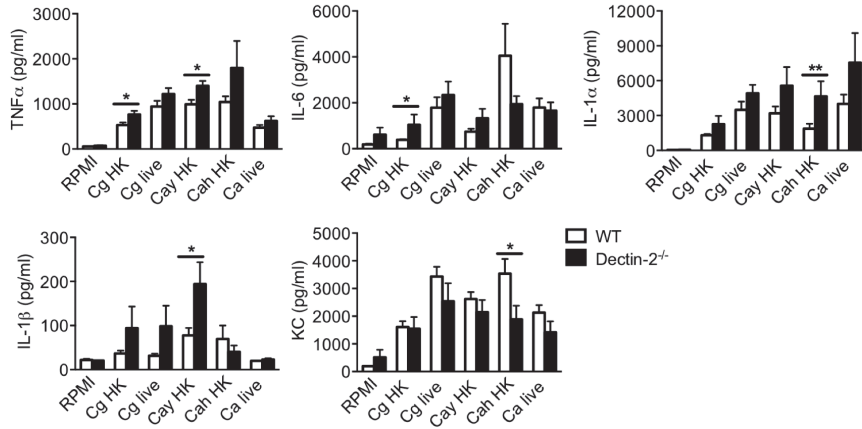
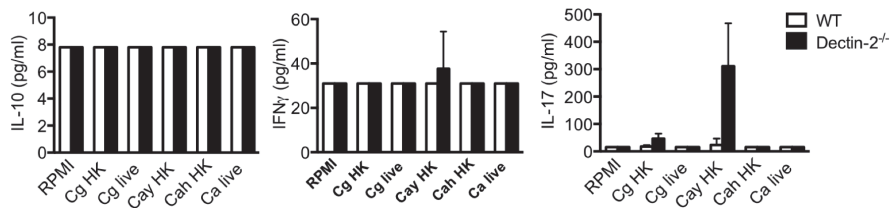
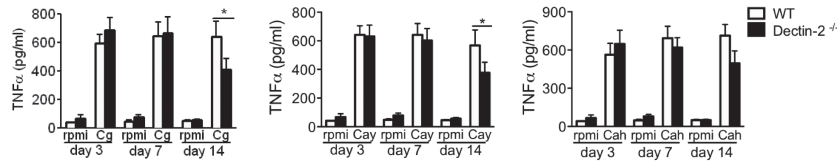
A macrophages**B** splenocytes

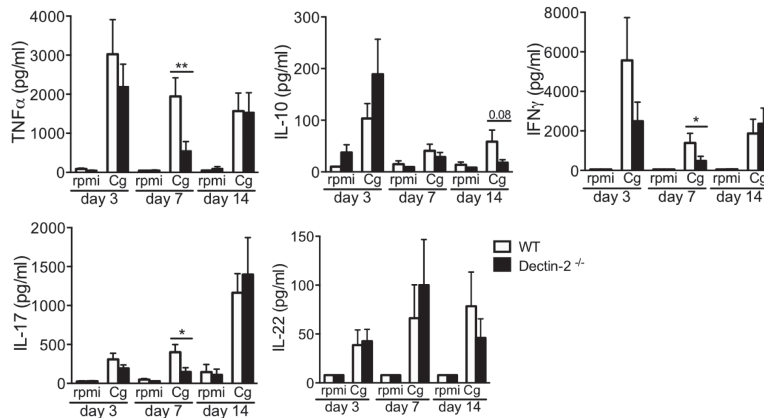
Fig. 3: Cytokine profile from naive mice. Cells from naive mice were stimulated for 24 h (peritoneal macrophages) (A) or for 48 h and 5 days (splenocytes) (B) with heat-killed (HK) or live *C. glabrata* (Cg), heat-killed *C. albicans* yeast (Cay) or hyphae (Cah) and live *C. albicans* (Ca). Levels of TNF-α, IL-6, KC, IL-1α, IL-1β, IL-10, IFN-γ and IL-17 accumulation were measured by ELISA. Data are represented as means ± SEM (n=8 mice per group). Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, $P < 0.05$ and **, $P < 0.01$.

the phagocytosis and subsequent killing of *C. glabrata* than Dectin-2^{-/-} macrophages (Fig. 5A). We further investigated the migration of peritoneal macrophages toward *C. glabrata* by live-cell video microscopy using a phagocytosis assay described previously (25). Dynamic analysis of individual macrophages, achieved by tracking cells to determine their directionality, distance, and velocity for the first hour of the assay, demonstrated no major differences in migration dynamics between macrophages harvested from wild-type or Dectin-2^{-/-} animals (Fig. 5B). However, in the same experiment, a complementary analysis revealed a significant difference between the two types of macrophages in terms of the uptake and phagocytosis of live *C. glabrata*. Following 30 min of cocubation with live *C. glabrata*, 51% of the wild-type macrophages had ingested at least one yeast, whereas Dectin-2^{-/-} macrophages showed a significantly reduced uptake of 34% (Fig. 5C). This significantly reduced uptake capacity was noticeable early during the phagocytosis assay (30 min and 60 min) but not at a later time point (180 min) (Fig. 5C). Additionally, the total numbers of fungal cells phagocytosed by macrophages from Dectin-2^{-/-} mice were also significantly lower than the numbers phagocytosed by wild-type macrophages

A macrophages



B splenocytes



C splenocytes

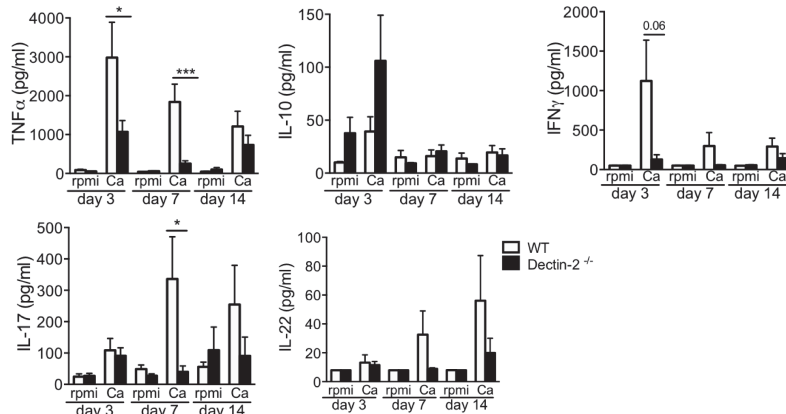


Fig. 4: Dectin-2 is required during systemic *C. glabrata* infection for subsequent splenocyte recall responses. Wild-type or Dectin-2^{-/-} mice received 10⁷ CFU of *C. glabrata* per mouse intravenously. After 3, 7 and 14 days, peritoneal macrophages (A) were restimulated *ex vivo* with either heat-killed *C. glabrata* (Cg), heat-killed *C. albicans* yeast (Cay) or hyphae (Cah) for 24 h. Levels of TNF-α accumulation in the supernatants was measured by ELISA. Results are means ± SEM (n=9-10 mice per group) from 2 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, P < 0.05 (A). Wild-type or Dectin-2^{-/-} mice received 10⁷ CFU of *C. glabrata*. Per mouse intravenously. After 3, 7 and 14 days, splenocytes (B, C) were restimulated *ex vivo* with either heat-killed *C. glabrata* (Cg) (B) or heat-killed *C. albicans* yeast (Ca) (C) for 48 h and 5 days. Levels of TNF-α, IL-10, IFN-γ (48 h), IL-17 and IL-22 (5 days) accumulation in the supernatants were measured by ELISA. Data are represented as means ± SEM (n=14-15 mice per group) from 3 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, P < 0.05; **, P < 0.01 and ***, P < 0.001.

at 30 min and 60 min of coincubation (Fig. 5D). Altogether, these results highlight a partial, yet significant role of Dectin-2 in mediating the uptake and engulfment of *C. glabrata* by macrophages. In addition, partial effects on fungal killing may also be envisioned, although more studies should be performed to establish that definitively.

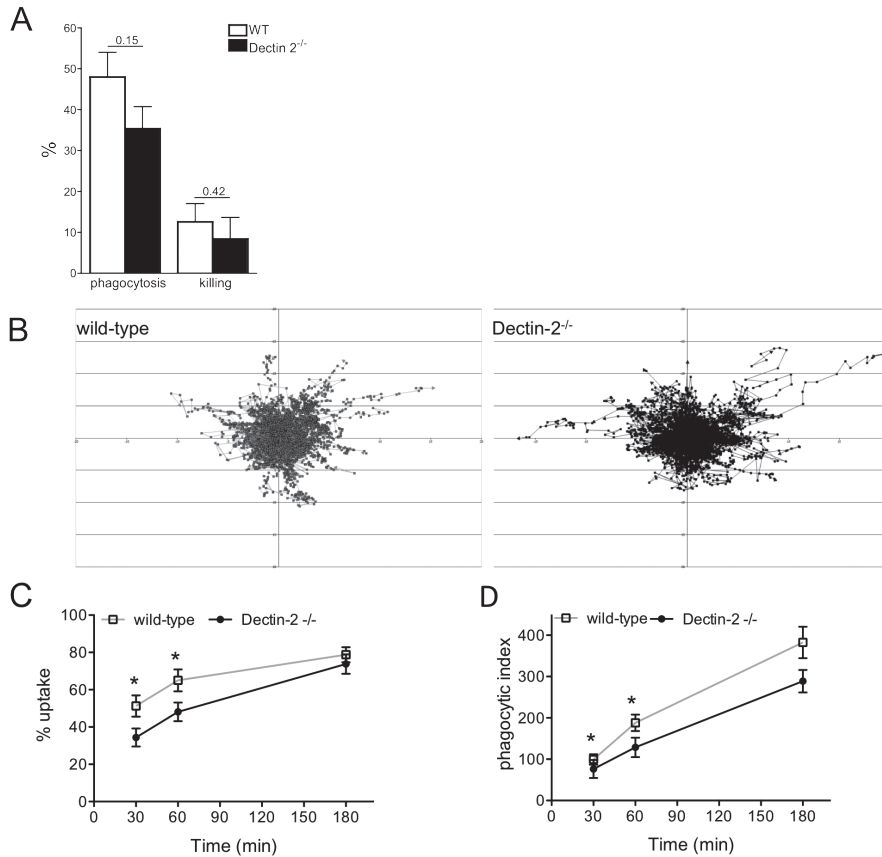


Fig. 5: Impact of Dectin-2 deficiency on phagocytosis and killing of *C. glabrata* by peritoneal macrophages. (A) Phagocytosis and killing of *C. glabrata* cells by murine peritoneal macrophages. The results are represented as percentage of phagocytosis (the percentage of fungal cells engulfed by macrophages present in the well) and percentage of killing (the percentage of killed fungal cells among the phagocytosed yeast). The *C. glabrata*/macrophage ratio was 10:1. Data are represented as means \pm SEM (n=5 mice per group). (B) Migration of peritoneal macrophages using live-cell video microscopy. Tracking diagrams illustrating the distances travelled, directionality and velocity of macrophages exposed to *C. glabrata* at a ratio of 3:1 fungal particles:macrophage. Due to the large number of macrophages tracked per video, the data were filtered to show only macrophages whose tracks lasted up until 30 min to 60 min, as previous work has shown that migration activity is greater early on [25]. Tracks represent the movement of individual macrophages relative to their starting position, symbols indicate the location of macrophages at 1 min intervals and arrows represent directionality. (n=7 control mice, and n=8 Dectin-2^{-/-} mice for 4 independent experiments). Percentage of uptake (C) and phagocytic index (D) using live-cell video microscopy. The percentage of macrophages that have ingested at least one *C. glabrata* yeast and the number of *C. glabrata* yeasts per 100 macrophages are depicted. *, $P < 0.05$.

Dectin-2^{-/-} neutrophils are less efficient in phagocytosing and killing *C. glabrata* *ex vivo*

We next assessed the recognition and killing of *C. glabrata* by neutrophils, pivotal players in the clearance of invading fungal cells from tissues. Neutrophils were harvested from the peritoneal cavity of both wild-type and Dectin-2^{-/-} mice and further subjected to an *ex vivo* phagocytosis and killing assay (23). The phagocytosis of *C. glabrata* by the Dectin-2^{-/-} neutrophils was considerably less effective than the phagocytosis of *C. glabrata* by neutrophils of wild-type mice (Fig. 6A). The killing of *C. glabrata* by the Dectin-2^{-/-} neutrophils was slightly diminished compared with the killing by control neutrophils (Fig. 6A). Importantly, neutrophils from Dectin-2^{-/-} mice showed a defect in secretion of ROS upon interaction with either live opsonized *C. glabrata* or *C. albicans* compared with the ROS secretion of wild-type neutrophils (Fig. 6B). Interestingly, the defect was specific for fungi, as no differences in ROS production were observed when cells were stimulated with PMA (Fig. 6B). Thus, Dectin-2 is important in both the uptake of *C. glabrata* by neutrophils and the subsequent ROS production and killing of the yeast.

4

Discussion

Dectin-2 is one of the main pattern recognition receptors for fungal mannans, with recent studies demonstrating an important role for this receptor in host defense against *C. albicans* (13). In the present study, we show that Dectin-2 is also an important component of the host defense against *C. glabrata*. The increased susceptibility of Dectin-2^{-/-} mice to a systemic

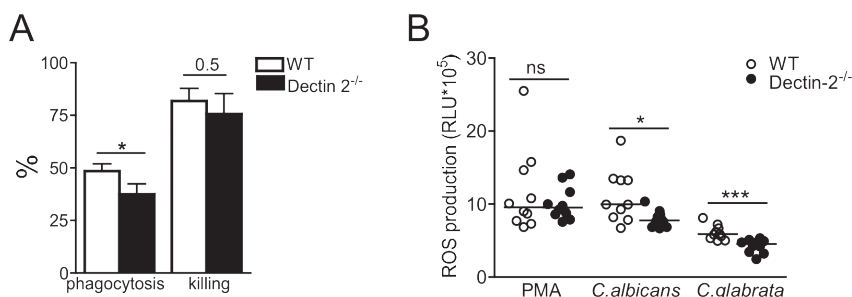


Fig. 6: Impact of Dectin-2 deficiency on phagocytosis and killing of *C. glabrata* by neutrophils. (A) Phagocytosis and killing of *C. glabrata* cells by murine neutrophils. The results are represented as percentage of phagocytosis (the percentage of fungal cells engulfed by neutrophils present in the well) and percentage of killing (the percentage of killed fungal cells among the phagocytosed yeast). The *C. glabrata*/neutrophil ratio was 10:1. Values represent means \pm SEM (n=10 mice per group) for 2 independent experiments. *P* values are relative to phagocytosis or killing induced by wild-type and Dectin-2^{-/-} macrophages. *, *P* < 0.05. (B) Dectin-2 neutrophils cannot induce the respiratory burst at the same extent as wild-type. Dectin-2^{-/-} or wild-type neutrophils were stimulated with RPMI, PMA, live opsonized *C. glabrata* or live opsonized *C. albicans*, and overall oxygen radical production (area under the curve after 60 min) was measured with luminal-enhanced chemiluminescence in isolated neutrophils. Data are represented as means \pm SEM (n=10 mice per group) for 2 independent experiments. Significance was determined with Mann-Whitney U-test. Statistically different groups are indicated as *, *P* < 0.05 and ***, *P* < 0.001. PMA: phorbol 12-myristate 13-acetate.

infection with *C. glabrata* is associated with a combination of decreased production of protective T helper-derived cytokines and defective phagocytosis and killing of the yeast by both neutrophils and macrophages.

Little is known of the role of the C-type lectin receptor, Dectin-2. A recent study reported that Dectin-2^{-/-} mice are highly susceptible to a systemic infection with *C. albicans* (13). Moreover, it has been shown that Dectin-2 plays an important role in the induction of protective Th17 responses (17) and that, together with Dectin-3, it forms a heterodimeric pattern recognition receptor for host defense against *C. albicans* (27). The intracellular signaling induced by Dectin-2 involves activation of CARD9 (28), phospholipase C-γ (29), and protein kinase C-σ (30). Our study demonstrates that Dectin-2 has a nonredundant role for host defense against *C. glabrata*. This is an important observation not only for our improved understanding of *C. glabrata* infection but also for Dectin-2 biology: while Dectin-2 has been suggested to recognize mainly *C. albicans* hyphae (13), its importance for recognition of a *Candida* species that does not usually develop hyphae is a broadening of the biological importance of Dectin-2. The increased susceptibility to *C. glabrata* was demonstrated in two independently generated groups of Dectin-2^{-/-} mice, reinforcing this conclusion. However, these mice were less susceptible to *C. glabrata* than to *C. albicans*, as reported earlier (13). The increased susceptibility of Dectin-2^{-/-} mice to *C. glabrata* was apparent through their incapacity to eliminate the fungus from the kidneys, the target organ of disseminated candidiasis (31). Previously reported studies already showed that systemic candidiasis increased the fungal burden in kidneys of Dectin-1^{-/-} mice compared with the fungal burden in kidneys of the wild-type mice (12). The fact that the animals were able to eradicate *C. glabrata* from the liver, in comparison with the results for the kidneys, confirms recent findings concerning organ-specific *Candida* infection and immune responses mediated primarily by neutrophils and monocytes, as well as by various molecular factors (32–34).

Fungal pathogens have developed a number of strategies to improve their chance of survival in the host environment; e.g., *Candida albicans* and *Cryptococcus neoformans* either destroy macrophages or escape via nonlytic exocytosis (35, 36). Interestingly, *C. glabrata* engulfed by macrophages does not undergo a morphological transition but survives and multiplies within mammalian macrophages (37, 38), which are ultimately damaged by ingested *C. glabrata* (38). In our study, we assessed the role of Dectin-2 for the phagocytosis and killing of *C. glabrata* and observed that neutrophils and macrophages lacking this receptor were less capable of ingesting and killing the yeast. Using live-cell video microscopy, we found that significantly fewer Dectin-2^{-/-} macrophages took up *C. glabrata*, in accordance with a lower phagocytic index where phagocytosis had occurred. These defects in the uptake and phagocytosis of *C. glabrata* could not be attributed to a defect in mobility. Finally, signaling through C-type lectin receptors induces cytokines and ROS that both activate the spleen tyrosine kinase (Syk)-caspase recruitment domain family member 9 (CARD9)-nuclear factor-κB (NF-κB) pathway (39) and, most likely, induce the intraphagosomal damage of fungal cells. In line with a role of the CARD9 pathway, Drewniak *et al.* showed that human CARD9 deficiency resulted in a selective defect in

the host defense against invasive fungal infection, caused by impaired phagocyte killing in unopsonized samples compared with the phagocyte killing in opsonized samples (40). In the present study, Dectin-2^{-/-} neutrophils produced less ROS than wild-type neutrophils after stimulation of cells with either opsonized *C. glabrata* or *C. albicans*, suggesting a role of Dectin-2 in the killing of *Candida* species despite the presence of opsonins.

The induction of proinflammatory cytokines is an important component of anti-*Candida* host defense. Macrophage-derived proinflammatory cytokines like IL-1, KC, or IL-6 activate the recruitment of phagocytes and the phagocytosis and killing of fungi by phagocytes, and mice deficient in these cytokines have an increased susceptibility to systemic candidiasis (41, 42). No major defects in the production of these cytokines were observed in cells isolated from *Candida*-challenged Dectin-2^{-/-} mice. In contrast, the secretion of cytokines produced by Th1 and Th17 lymphocytes was significantly decreased in Dectin-2^{-/-} mice. IFN- γ is the main product of Th1 lymphocytes, and it has been proven to have a crucial role in antifungal host defense in mice (43). The defective production of IFN- γ by the splenocytes of Dectin-2^{-/-} mice may represent one of the mechanisms determining the increased susceptibility to *C. glabrata* in these mice. Similarly, the Th17 cytokine, IL-17, represents an important component of antifungal host defense through its activation of neutrophils (44), while patients with defective Th17 responses develop chronic mucocutaneous candidiasis (45). The level of IL-17 produced by Dectin-2^{-/-} splenocytes was significantly decreased at day 7 postinfection, suggesting a role for Dectin-2 in regulating splenocyte recall responses to fungal infection. The defective Th17 responses in Dectin-2^{-/-} mice could therefore contribute to their susceptibility to *C. glabrata*. These results underline the conclusions of Saijo *et al.* (13) concerning Dectin-2-mediated immunity to *C. albicans*. The IL-17 defect in response to *C. glabrata* observed in our study was, however, less pronounced than that previously reported for *C. albicans* (13). Moreover, we should observe that the cytokine differences were most pronounced earlier during infection. At later time points during the infection, the picture starts to be biased by the higher fungal burden in the KO mice, which may counterbalance the defective cytokine production capacity.

The known fungal ligands recognized by Dectin-2 are α -mannans (13). Whereas significant research has been conducted on the structure of the *C. albicans* cell wall, much less is known regarding the cell wall components of *C. glabrata*. While recognition of mannans of *C. glabrata* by Dectin-2 is most likely, future studies should define the precise ligand-receptor interaction profile, especially as *C. glabrata* is less likely to form hyphae than *C. albicans*. Moreover, both endogenous (T cell) and exogenous (microbial carbohydrate) ligands for Dectin-2 may be important for the generation and maintenance of tolerance or the recognition of carbohydrate structures. Of particular interest is that previous reports have highlighted the ability of Dectin-2 to specifically signal after recognition of the hyphal form of *C. albicans* (28). The interaction between *C. glabrata* and Dectin-2 is different, as *C. glabrata* does not form traditional compartmentalized hyphae. Still, previous reports have described tubes and pseudohypha formation in several clinical isolates of *C. glabrata* (46), and these might be recognized by Dectin-2.

In conclusion, we demonstrate that the immune responses triggered by Dectin-2 during infection with *C. glabrata* might link innate immune recognition, phagocytosis, and killing of the fungus with the adaptive T helper-dependent responses. A combination of these mechanisms controls *C. glabrata* during systemic infection. Deciphering the precise mechanisms responsible for host defense against *C. glabrata* represents an important step in understanding of the pathophysiology of the disease and for the possibility to design future novel immunotherapeutic strategies.

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References

- 1 Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M. Candida bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008-2009). *Int J Antimicrob Agents*. [Research Support, Non-U.S. Gov't]. 2011 Jul;38(1):65-9.
- 2 Abi-Said D, Anaissie E, Uzun O, Raad I, Pinzcowski H, Vartivarian S. The epidemiology of hematogenous candidiasis caused by different Candida species. *Clin Infect Dis*. 1997 Jun;24(6):1122-8.
- 3 Kauffman CA, Vazquez JA, Sobel JD, Gallis HA, McKinsey DS, Karchmer AW, et al. Prospective multicenter surveillance study of funguria in hospitalized patients. The National Institute for Allergy and Infectious Diseases (NIAID) Mycoses Study Group. *Clin Infect Dis*. 2000 Jan;30(1):14-8.
- 4 Ruan SY, Chu CC, Hsueh PR. In vitro susceptibilities of invasive isolates of Candida species: rapid increase in rates of fluconazole susceptible-dose dependent Candida glabrata isolates. *Antimicrob Agents Chemother*. 2008 Aug;52(8):2919-22.
- 5 Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, et al. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol*. 2004 Apr;42(4):1519-27.
- 6 Fidel PL, Jr., Vazquez JA, Sobel JD. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin Microbiol Rev*. 1999 Jan;12(1):80-96.
- 7 Anaissie EJ, Vartivarian SE, Abi-Said D, Uzun O, Pinzcowski H, Kontoyiannis DP, et al. Fluconazole versus amphotericin B in the treatment of hematogenous candidiasis: a matched cohort study. *Am J Med*. 1996 Aug;101(2):170-6.
- 8 Goodman JL, Winston DJ, Greenfield RA, Chandrasekar PH, Fox B, Kaizer H, et al. A controlled trial of fluconazole to prevent fungal infections in patients undergoing bone marrow transplantation. *N Engl J Med*. 1992 Mar 26;326(13):845-51.
- 9 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat Rev Microbiol*. 2008 Jan;6(1):67-78.
- 10 Poulain D, Jouault T. Candida albicans cell wall glycans, host receptors and responses: elements for a decisive crosstalk. *Curr Opin Microbiol*. 2004 Aug;7(4):342-9.
- 11 Cambi A, Netea MG, Mora-Montes HM, Gow NA, Hato SV, Lowman DW, et al. Dendritic cell interaction with Candida albicans critically depends on N-linked mannan. *J Biol Chem*. 2008 Jul 18;283(29):20590-9.
- 12 Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol*. 2007 Jan;8(1):31-8.
- 13 Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. *Immunity*. 2010 May 28;32(5):681-91.
- 14 Ariizumi K, Shen GL, Shikano S, Ritter R, 3rd, Zukas P, Edelbaum D, et al. Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J Biol Chem*. 2000 Apr 21;275(16):11957-63.
- 15 McGreal EP, Rosas M, Brown GD, Zamze S, Wong SY, Gordon S, et al. The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology*. 2006 May;16(5):422-30.
- 16 Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol*. 2009 Jan 15;182(2):1119-28.
- 17 Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med*. 2009 Aug 31;206(9):2037-51.
- 18 Sato K, Yang XL, Yudate T, Chung JS, Wu J, Luby-Phelps K, et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J Biol Chem*. 2006 Dec 15;281(50):38854-66.
- 19 Gavino AC, Chung JS, Sato K, Ariizumi K, Cruz PD, Jr. Identification and expression profiling of a human C-type lectin, structurally homologous to mouse dectin-2. *Exp Dermatol*. 2005 Apr;14(4):281-8.
- 20 Zhang Z, Lutz B. Cre recombinase-mediated inversion using lox66 and lox71: method to

- introduce conditional point mutations into the CREB-binding protein. *Nucleic Acids Res.* 2002 Sep 1;30(17):e90.
- 21 Koszul R, Malpertuy A, Frangeul L, Bouchier C, Wincker P, Thierry A, et al. The complete mitochondrial genome sequence of the pathogenic yeast *Candida (Torulopsis) glabrata*. *FEBS Lett.* 2003 Jan 16;534(1-3):39-48.
 - 22 Kullberg BJ, van 't Wout JW, van Furth R. Role of granulocytes in increased host resistance to *Candida albicans* induced by recombinant interleukin-1. *Infect Immun.* 1990 Oct;58(10):3319-24.
 - 23 Kullberg BJ, van 't Wout JW, Hoogstraten C, van Furth R. Recombinant interferon-gamma enhances resistance to acute disseminated *Candida albicans* infection in mice. *J Infect Dis.* 1993 Aug;168(2):436-43.
 - 24 McKenzie CG, Koser U, Lewis LE, Bain JM, Mora-Montes HM, Barker RN, et al. Contribution of *Candida albicans* cell wall components to recognition by and escape from murine macrophages. *Infect Immun.* 2010 Apr;78(4):1650-8.
 - 25 Lewis LE, Bain JM, Okai B, Gow NA, Erwig LP. Live-cell video microscopy of fungal pathogen phagocytosis. *J Vis Exp.* 2013(71).
 - 26 Versleijen MW, Oyen WJ, Roelofs HM, van Emst-de Vries SE, Willems PH, Jansen JB, et al. Immune function and leukocyte sequestration under the influence of parenteral lipid emulsions in healthy humans: a placebo-controlled crossover study. *Am J Clin Nutr.* 2008 Mar;87(3):539-47.
 - 27 Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, et al. C-Type Lectin Receptors Dectin-3 and Dectin-2 Form a Heterodimeric Pattern-Recognition Receptor for Host Defense against Fungal Infection. *Immunity.* 2013 Aug 22;39(2):324-34.
 - 28 Bi L, Gojestani S, Wu W, Hsu YM, Zhu J, Ariizumi K, et al. CARD9 mediates dectin-2-induced I κ B kinase ubiquitination leading to activation of NF- κ B in response to stimulation by the hyphal form of *Candida albicans*. *J Biol Chem.* 2010 Aug 20;285(34):25969-77.
 - 29 Gorjestani S, Yu M, Tang B, Zhang D, Wang D, Lin X. Phospholipase C γ 2 (PLC γ 2) is key component in Dectin-2 signaling pathway, mediating anti-fungal innate immune responses. *J Biol Chem.* 2011 Dec 23;286(51):43651-9.
 - 30 Strasser D, Neumann K, Bergmann H, Marakalala MJ, Guler R, Rojowska A, et al. Syk kinase-coupled C-type lectin receptors engage protein kinase C- σ to elicit Card9 adaptor-mediated innate immunity. *Immunity.* 2012 Jan 27;36(1):32-42.
 - 31 Spellberg B, Ibrahim AS, Edwards JE, Jr., Filler SG. Mice with disseminated candidiasis die of progressive sepsis. *J Infect Dis.* 2005 Jul 15;192(2):336-43.
 - 32 Lionakis MS, Lim JK, Lee CC, Murphy PM. Organ-specific innate immune responses in a mouse model of invasive candidiasis. *J Innate Immun.* [Research Support, N.I.H., Intramural]. 2011;3(2):180-99.
 - 33 Lionakis MS, Fischer BG, Lim JK, Swamydas M, Wan W, Richard Lee CC, et al. Chemokine receptor Ccr1 drives neutrophil-mediated kidney immunopathology and mortality in invasive candidiasis. *PLoS Pathog.* [Research Support, N.I.H., Intramural]. 2012;8(8):e1002865.
 - 34 Research Support, Non-U.S. Gov't]. 2012;8(8):e1002865.
 - 35 Ngo LY, Kasahara S, Kumasaka DK, Knoblauch SE, Jhingran A, Hohl TM. Inflammatory Monocytes Mediate Early and Organ-Specific Innate Defense During Systemic Candidiasis. *J Infect Dis.* 2013 Aug 28.
 - 36 Alvarez M, Casadevall A. Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Curr Biol.* 2006 Nov 7;16(21):2161-5.
 - 37 Bain JM, Lewis LE, Okai B, Quinn J, Gow NA, Erwig LP. Non-lytic expulsion/exocytosis of *Candida albicans* from macrophages. *Fungal Genet Biol.* 2012 Sep;49(9):677-8.
 - 38 Kaur R, Ma B, Cormack BP. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci U S A.* 2007 May 1;104(18):7628-33.
 - 39 Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, et al. The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. *J Immunol.* 2011 Sep 15;187(6):3072-86.
 - 40 Drummond RA, Saijo S, Iwakura Y, Brown GD. The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. *Eur J Immunol.* 2011 Feb;41(2):276-81.
 - 41 Drewniak A, Gazendam RP, Tool AT, van Houdt M, Jansen MH, van Hamme JL, et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood.* [Case Reports

- 42 Research Support, Non-U.S. Gov't]. 2013 Mar 28;121(13):2385-92.
- 43 Basu S, Quilici C, Zhang HH, Grail D, Dunn AR. Mice lacking both G-CSF and IL-6 are more susceptible to *Candida albicans* infection: critical role of neutrophils in defense against *Candida albicans*. *Growth Factors*. 2008 Feb;26(1):23-34.
- 44 Gorjestani S, Darnay BG, Lin X. Tumor necrosis factor receptor-associated factor 6 (TRAF6) and TGFbeta-activated kinase 1 (TAK1) play essential roles in the C-type lectin receptor signaling in response to *Candida albicans* infection. *J Biol Chem*. 2012 Dec 28;287(53):44143-50.
- 45 Zhou P, Sieve MC, Bennett J, Kwon-Chung KJ, Tewari RP, Gazzinelli RT, et al. IL-12 prevents mortality in mice infected with *Histoplasma capsulatum* through induction of IFN-gamma. *J Immunol*. 1995 Jul 15;155(2):785-95.
- 46 Dejima T, Shibata K, Yamada H, Hara H, Iwakura Y, Naito S, et al. Protective role of naturally occurring interleukin-17A-producing gammadelta T cells in the lung at the early stage of systemic candidiasis in mice. *Infect Immun*. 2011 Nov;79(11):4503-10.
- 47 van de Veerdonk FL, Plantinga TS, Hoischen A, Smekens SP, Joosten LA, Gilissen C, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. *N Engl J Med*. 2011 Jul 7;365(1):54-61.
- 48 Lachke SA, Joly S, Daniels K, Soll DR. Phenotypic switching and filamentation in *Candida glabrata*. *Microbiology*. 2002 Sep;148(Pt 9):2661-74.

PART TWO

**Modulation of innate immune
responses after *Candida* infection:
induction of trained immunity**

5

**Trained immunity or tolerance:
opposing functional programs induced
in human monocytes after engagement
of various pattern recognition receptors**

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Abstract

Upon priming with *Candida albicans* or with the fungal cell wall component β -glucan, monocytes respond with an increased cytokine production upon restimulation, a phenomenon termed “trained immunity.” In contrast, the prestimulation of monocytes with lipopolysaccharide has long been known to induce tolerance. Because the vast majority of commensal microorganisms belong to bacterial or viral phyla, we sought to systematically investigate the functional reprogramming of monocytes induced by the stimulation of pattern recognition receptors (PRRs) with various bacterial or viral ligands. Monocytes were functionally programmed for either enhanced (*training*) or decreased (*tolerance*) cytokine production, depending on the type and concentration of ligand they encountered. The functional reprogramming of monocytes was also associated with cell shape, granulocytosis, and cell surface marker modifications. The training effect required p38- and Jun N-terminal protein kinase (JNK)-mediated mitogen-activated protein kinase (MAPK) signaling, with specific signaling patterns directing the functional fate of the cell. The long-term effects on the function of monocytes were mediated by epigenetic events, with both histone methylation and acetylation inhibitors blocking the training effects. In conclusion, our experiments identify the ability of monocytes to acquire adaptive characteristics after prior activation with a wide variety of ligands. Trained immunity and tolerance are two distinct and opposing functional programs induced by the specific microbial ligands engaging the monocytes.

Keywords: trained immunity, microbial ligands, monocytes, memory.

Introduction

More than 3 decades ago, a number of studies reported enhanced immune responses upon reinfection in diverse invertebrate taxa that do not possess adaptive immunity, such as cockroaches (1–3), shrimp (4, 5), and mealworm beetles (6). Interestingly, invertebrates also have proven to be able to mount enhanced secondary immune responses (7) and to transmit protection to their offspring (8). While this protection is nonspecific, several studies demonstrated that these effects enable discrimination between different classes of pathogens. Recently, Witteveldt *et al.* showed that such immune memory can be used in the vaccination of invertebrates (9). Whereas vertebrates use somatic rearrangement of immunological receptors to induce adaptive immune responses, one mechanism employed by the host defenses of invertebrates to confer adaptation to infection is alternative splicing of pattern recognition genes, such as Down syndrome cell adhesion molecule, which generates a highly diverse set of >31,000 potential alternative splice forms (10, 11). These two molecular processes have the same consequence: they create a receptor repertoire that is sufficiently diverse for discriminating between the broad varieties of different antigens.

The function of prototypic mammalian innate immune cells, such as NK cells, can also be enforced, leading to protection against reinfection with viral pathogens (12–15). Similarly, monocytes and/or macrophages exhibit memory characteristics that mediate protective effects after a second encounter with a pathogen (16, 17). We have termed this phenomenon “trained immunity,” defined as enhanced nonspecific innate immune protection that is suggested to be mediated by epigenetic mechanisms (18). In contrast to trained immunity, engagement of the Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) has been known for several decades to inhibit the cell function in a process called LPS-induced tolerance (19, 20). LPS tolerance is an active process involving epigenetic remodeling (21), and this process has been suggested to be the basic mechanism responsible for the immunoparalysis that occurs after Gram-negative sepsis (22). Despite the importance of both LPS tolerance (19, 20) and trained immunity (16, 17), very little is known about the modulatory characteristics of the various classes of pattern recognition receptors and microbial ligands.

The aim of this study was to systematically investigate the potential to either train or induce tolerance of the well-known classes of PRRs expressed on monocytes/macrophages, as depicted in Table 1. We demonstrate that several bacterial, fungal, and viral ligands induce the functional reprogramming of monocytes, leading to either nonspecific enhanced (*training*) or diminished (*tolerance*) cytokine production upon secondary stimulation, a process that is often dependent on ligand concentration. Understanding the nature of the signaling pathways in determining the functional fate of innate host responses upon sequential stimulation of pattern recognition receptors may represent an important step toward understanding bacterial and fungal colonization and/or invasion of the mucosa on one hand, and for developing novel immunotherapeutic strategies on the other hand.

Table 1: Pattern recognition receptors, microbial ligands and their adapter molecules.

PRR	Microbial ligands	Adapter molecules
Membrane receptors		
Dectin-1	β -glucan	CARD9
TLR2	lipoteichoic acid (Pam3CSK4) lipoproteins glycosyl-phosphatidylinositols from <i>Toxoplasma gondii</i>	TIRAP MyD88
TLR4	lipopolysaccharide (LPS) envelope proteins of RSV glycosyl-phosphatidylinositols from <i>Toxoplasma gondii</i>	TIRAP MyD88 TRAM TRIF
TLR5	Flagellin	MyD88
Endosomal receptors		
TLR3	dsRNA polyinosinic:polycytidylic acid (Poly (I:C))	TRIF
TLR7/8	ssRNA (R848)	MyD88
TLR9	CpG DNA	MyD88
Cytosolic receptors		
NOD1	L-Ala- γ -D-Glu-mDAP (Tri-DAP)	CARD6
NOD2	Muramyl dipeptide (MDP)	CARD12

Materials and Methods

Blood samples. Blood was collected from healthy volunteers at Sanquin Bloodbank in Nijmegen, Netherlands. Informed consent was obtained from the volunteers included. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats.

Reagents. Culture medium used was Roswell Park Memorial Institute (RPMI) 1640 Dutch modifications from Sigma-Aldrich, supplemented with 1% gentamicin, 1% L-glutamine and 1% pyruvate (Life Technologies, Nieuwekerk, Netherlands). *Candida* β -glucan was isolated and purified as previously described (23). Other reagents were obtained as follows: Pam3CSK4 (EMC microcollections, L2000), LPS (*E. coli* serotype 055:B5, Sigma-Aldrich) with an additional purification step (24), Flagellin from *Salmonella typhimurium* FLA-ST, CpG type C ODN M362, Tri-DAP and R848 (InvivoGen), Poly(I:C) (Brunswick, USA), Syk inhibitor (EMD, 574711), P38-inhibitor SB202190 (Sigma-RBI, S7067), ERK inhibitor (Promega, V1121) and JNK inhibitor SP600125 (AG Scientific, Inc., S-2022), PMA (phorbol 12-myristate 13-acetate; Sigma), CD45-PC7 (Beckman Coulter), CD68-APC (Biolegend), CD14-PE (Beckman Coulter), CD11b-FITC (Beckman Coulter) and

DC-SIGN (Biolegend). Versene solution was purchased from InVitrogen and the Cytotox96 nonradioactive assay was purchased from Promega. Syk- inhibitor was used previously, as described (16). The epigenetic inhibitors, used previously (16, 17), were as follows: histone methyltransferase inhibitor (MTA, Sigma-Aldrich, D5011), epigallocatechin-3-gallate histone acetyltransferase inhibitor (HAT-i) (EGCG, Sigma, E4143), histone demethylase inhibitor (Pargyline, Sigma-Aldrich, P8013) and histone deacetylase inhibitor (ITF2357/9, ITALFARMACO S.p.A as described in patent WO 97/43251, US 6034096 (25, 26)).

Stimulation experiments. Mononuclear cells were isolated by density centrifugation of PBS-diluted blood (dilution 1:1) over a Ficoll-Paque PLUS gradient (GE Healthcare). PBMCs were washed 3 times with PBS and suspended in RPMI 1640 culture medium supplemented with 1% gentamicin, 1% pyruvate, and 1% L-glutamine. Cells were counted in a Coulter counter (Coulter Electronics), and the concentration was adjusted to 5×10^6 cells/ml. For stimulation experiments, a 100 μ l suspension of 5×10^5 PBMCs in RPMI medium was added to flat-bottomed 96-well plates (Greiner Bio-One) and incubated for 1 h at 37°C, with 5% CO₂. Adherent monocytes were selected by three times washing out non-adherent cells with warm PBS. For training, cells were preincubated for 24 h with either RPMI (negative control), β -glucan (1 μ g/ml) (positive control), or with serial dilutions of Pam3CSK4 (100 μ g/ml to 0.01 pg/ml), LPS (100 ng/ml to 10⁻⁵ pg/ml), Flagellin (10 μ g/ml to 0.01 pg/ml), Poly(I:C) (100 μ g/ml to 0.01 pg/ml), R848 (100 μ g/ml to 0.01 pg/ml), CpG (10 μ g/ml to 0.001pg/ml), Tri-DAP (10 μ g/ml to 0.001 pg/ml), and MDP (10 μ g/ml to 0.01 pg/ml) for 24 h. After the first incubation, monocytes were washed with warm PBS and maintained in RPMI supplemented with 10% pooled human serum for 5 days (media was refreshed at day 3). Thereafter, the cells were subjected to a second stimulation of cytokine production with LPS (10 ng/ml), Pam3CSK4 (10 μ g/ml) or RPMI (negative control). After 24 h, duplicate supernatants were collected, pooled and stored at -20°C until assayed.

To investigate whether various inhibitors would affect training, adherent monocytes were preincubated for 1 h, prior to the first stimulation, with p38 inhibitor (1 μ M), Syk inhibitor (140 μ M), JNK inhibitor (20 μ M), ERK inhibitor (10 μ M), MTA (1 mM), EGCG (15 μ M), pargyline (3 μ M) or ITF2357/9 (100 nM). Subsequently, the microbial stimuli were added to the cells together with the inhibitors for an additional 24 h. Thereafter, the cells were washed with PBS and further incubated in culture media supplemented with 10% pooled human serum for 5 days. On day 6, the trained macrophages were subjected to a second stimulation with either LPS or Pam3CSK4, for 24 h. The supernatants were collected and stored at -20°C until assessed.

Cytokine assay. The concentrations of TNF- α (R&D Systems, Abingdon, UK) and IL-6 (Sanquin, Amsterdam, Netherlands) were measured 24 h after the second stimulation, in cell culture supernatants using ELISA, according to the manufacturer instructions.

Lactate hydrogenase (LDH) were performed with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), according to the manufacturer's instructions. Briefly, the adherent monocytes were incubated for 24 h with different microbial ligands and the secreted LDH was measured in cell culture supernatants. To check for putative cytotoxic effects of

MAPK-, Syk- and epigenetics inhibitors, the adherent monocytes were incubated for 24 h with RPMI, MAPK kinase-, Syk- or epigenetic inhibitors. Thereafter, the supernatants and cells were collected and stored until assessed. The values of inhibitors in RPMI were considered as background and subsequently subtracted from the final results. RPMI was considered negative control. The final values are depicted in %.

Extracellular Cytokine Staining and Flow Cytometry. 3 ml of PBMCs with a concentration of 5×10^6 cells/ml were cultured in a well of a 6-well plate. After 1 h of incubation at 37°C, with 5% CO₂, non-adherent cells were washed 3 times with warm PBS. The remaining adherent monocytes were prestimulated with concentrations of different ligands which would induce either training or tolerance, such as β -glucan (1 μ g/ml), MDP (1 μ g/ml), Flagellin (1 μ g/ml), R848 (1 μ g/ml), Pam3CSK4 (1 μ g/ml) and RPMI (negative control), for 24 h. The stimuli were washed away and the cells were further incubated for 5 days in the presence of RPMI supplemented with 10% pooled human serum. On day 7, the cells were incubated for 1 h with Versene solution, collected, harvested by centrifugation and suspended in PBS supplemented with 1% PBA. The cells were washed two times and stained extracellularly with the following antibodies: anti-CD45-PeCy7 (Beckman Coulter), anti-CD68-APC (Biolegend), anti-CD14-PE (Beckman Coulter), anti-CD11b-FITC (Beckman Coulter) and anti-DC-SIGN-APC (Biolegend). Samples were measured on a FACS FC500, and data were analyzed using the CXP software (Beckman Coulter).

Statistical analysis. IBM SPSS Statistics software, Version 20, was used to perform statistical analysis. All experiments were performed at least three times with minimum six volunteers. The data represent cumulative results of all experiments performed, and the number of donors is given as “n” in figure legends. In order to assess the training effect of a specific ligand upon a secondary stimulation, non-trained cells stimulated with LPS (or Pam3CSK4) were compared with trained cells restimulated with LPS (or Pam3CSK4). The differences between groups were analyzed with Wilcoxon signed rank test and were considered statistically significant at P values of < 0.05.

Results

The type of ligand-PRR interactions as well as pathogen associated molecular pattern (PAMP) concentration decides the inflammatory state of monocytes

Some microbial ligands such as β -1,3-glucan (β -glucan) and the muramyl dipeptide (MDP) component of peptidoglycans induce trained immunity through the engagement of dectin-1 and nucleotide-binding oligomerization domain 2 (NOD2), respectively (16, 17), while the TLR4 agonist, LPS, induces tolerance (19, 20). We performed a systematical assessment to determine whether exposure of adherent monocytes to the microbial ligands of the TLR and NOD-like receptor (NLR) classes of pattern recognition receptors such as Pam3CSK4, LPS, Flagellin, Poly(I:C), R848, CpG and Tri-DAP also influences the cell response to a secondary

stimulation. The adherent monocytes were therefore exposed to each different PRR ligand for 24 h (first stimulus) at a concentration that would induce an inflammatory response, followed by a washout step. Following a resting time of 5 days, period during which monocytes in the absence of any primary event differentiate into macrophages, cells were exposed to a secondary stimulation with either LPS or Pam3CSK4 (second stimulus) (Fig. 1A).

The *C. albicans* cell wall component β -glucan strongly trains monocytes, leading to an enhanced production of TNF- α and IL-6 upon nonspecific secondary stimulation with LPS or Pam3CSK4 (16); therefore, β -glucan preincubation of monocytes was used as an inherent control of effective training throughout this study (Fig. 1B). In contrast to the training obtained upon engagement of dectin-1, the stimulation of the membrane receptors TLR2, TLR4 and TLR5 by inflammatory doses of Pam3CSK4 (100 μ g/ml), LPS (100 ng/ml) and Flagellin (10 μ g/ml) induced a long-term tolerant state in which monocytes produced fewer proinflammatory cytokines (TNF- α and IL-6) upon restimulation than did the RPMI-treated control cells (Fig. 1C-1E). Although LPS tolerance has been described *in vitro* (21) and *in vivo* (27) in short-term experiments, no studies have assessed the long-term effects of LPS. Using our training model of human adherent monocytes, we described a long-term tolerance effect of LPS (100 ng/ml) on the cytokine production observed upon secondary stimulation. In contrast to the membrane associated-TLRs, engaging cytosolic PRRs during a primary exposure to pure ligands revealed more diverse outcomes in terms of innate immune memory features. Preactivation of the endosomal TLR3 with a strong inflammatory dose of Poly(I:C) (100 μ g/ml) maintained the cells in a tolerant status, being refractory to the second stimulation (Fig. 2A). Similarly, engagement of the endosomal TLR7/8 with R848 (100 μ g/ml) also induced a refractory state of monocytes for TNF- α but not for IL-6 (Fig. 2B). However, the use of CpG (10 μ g/ml) to preactivate TLR9 failed to induce any trained or tolerant effect (Fig. 2C). In contrast, engagement of NOD1 by Tri-DAP (10 μ g/ml) and NOD2 by MDP (10 μ g/ml) significantly trained the cells towards an increased production of TNF- α and IL-6 (Fig. 2D and E). Of note, while no correlation could be observed between the trained or refractory status obtained and the intracellular adaptor molecules of TLRs (i.e., MyD88, Toll-interleukin 1 receptor [TIR] domain-containing adaptor protein [TIRAP], and TIR domain-containing adapter-inducing beta interferon [TRIF]) (Table 1), it seemed that engaging receptors that signal through caspase recruitment domain (CARD) molecules favor an enhanced trained immunity status. Altogether, the activation of TLRs present at the cell surface seemed to maintain the cells in a refractory status. However, engaging endosomal TLRs had a striking and different effect on the functional fate of monocytes, regardless of the adaptor molecules used to signal. Finally, engaging cytosolic NLRs receptors seemed to result in an enhanced proinflammatory immune status.

In order to assess whether the ligand concentration would influence the training of monocytes, titrations of all the ligands assessed in this study were performed. None of the first incubations with low doses of ligands induced measurable amounts of proinflammatory cytokines in supernatants collected after the first 24 hours (data not shown). We first deciphered the titration response of the cell membrane-associated TLRs. Systematically

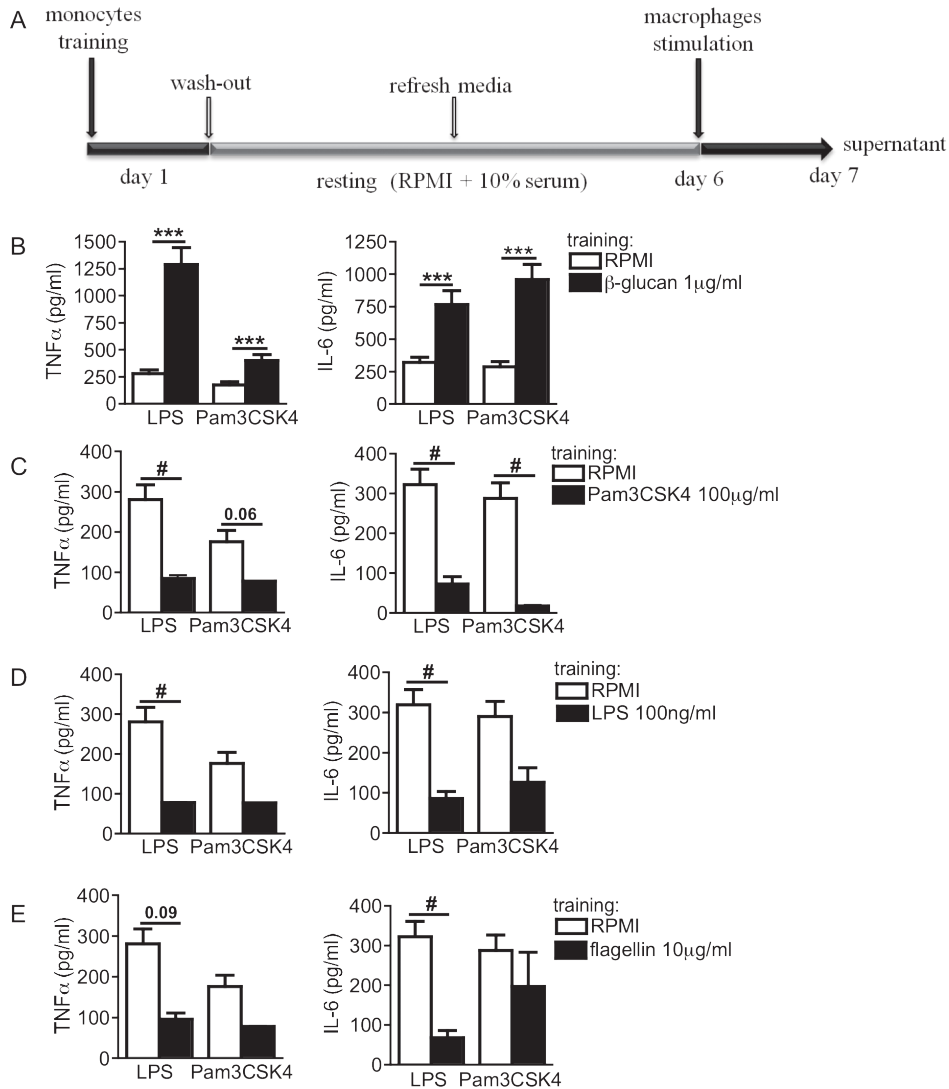


Fig. 1: (A) *In vitro* training scheme. Adherent monocytes were incubated with pure ligands (training stimuli) for 24 h at 37°C. Following the first incubation, the cells were washed with warm PBS and further incubated with RPMI and pooled human serum for 5 days. In the absence of any training stimulus during the first 24 h, primary monocytes maintained in culture for several days eventually differentiated into macrophages. After this resting period, different pattern recognition receptor ligands were added (second stimulus) for an additional 24 h. (B to E) Tolerance induced by the membrane receptors. The cells were preexposed for 24 h to culture medium, β -glucan (1 μ g/ml) (B), Pam3CSK4 (100 μ g/ml) (C), LPS (100 ng/ml) (D), or Flagellin (10 μ g/ml) (E). Primary stimuli were washed out, and after 6 days, the macrophages were restimulated with RPMI (negative control), LPS (10 ng/ml), or Pam3CSK4 (10 μ g/ml). While β -glucan induced training, Pam3CSK4, LPS, and flagellin induced tolerance. The data are presented as the means \pm SEM ($n > 8$, 5 independent experiments), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$ (tolerance). Wilcoxon signed rank test was used to detect significant differences.

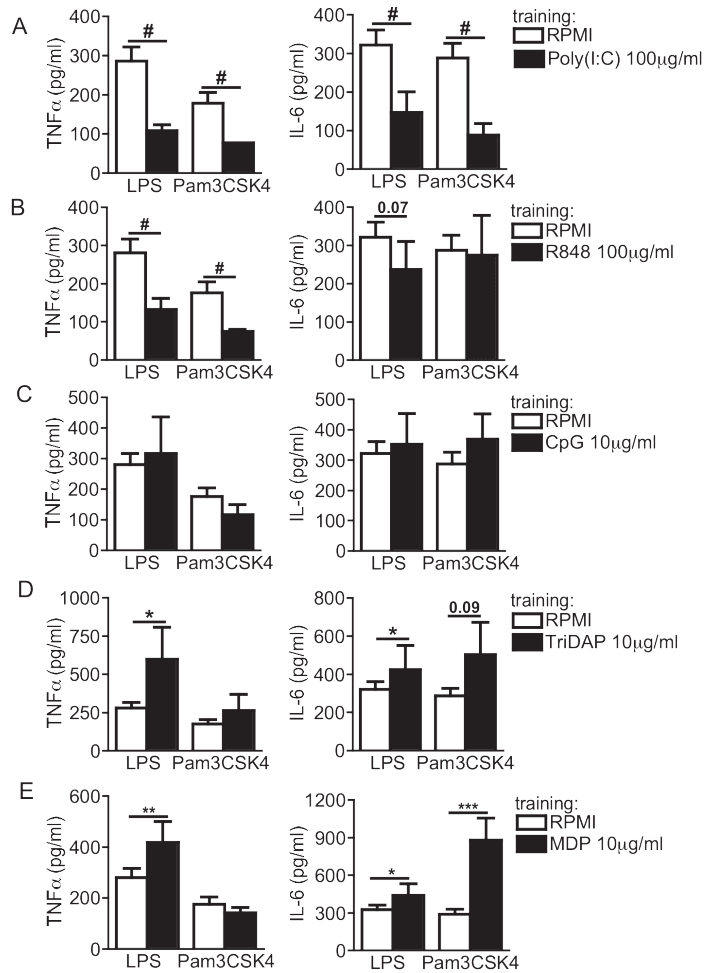


Fig. 2: Differential effects of endosomal and cytosolic receptors on adherent monocytes. The cells were preexposed for 24 h to culture medium, Poly(I:C) (100 μg/ml) (A), R848 (100 μg/ml) (B), CpG (10 μg/ml) (C), Tri-DAP (10 μg/ml) (D), and MDP (10 μg/ml) (E). Primary stimuli were washed out, and after 6 days, the macrophages were restimulated with RPMI (negative control), LPS (10 ng/ml), or Pam3CSK4 (10 μg/ml). While Poly(I:C) induced tolerance for TNF-α and IL-6, R848 was able to induce tolerance for TNF-α only, and CpG did not exert any significant effect on the inflammatory status of monocytes. Both types of NLRs were able to enhance the status of monocytes upon a second exposure to either LPS or Pam3CSK4. The data are presented as the means ± SEM ($n > 8$, 5 independent experiments), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, * $p < 0.05$ (tolerance). Wilcoxon signed rank test was used to detect significant differences.

lowering the amount of Pam3CSK4 which is used to activate the cell membrane associated TLR2, strongly modified the monocytes reprogramming characteristics. Pam3CSK4 in low concentration (1 μg/ml) resulted in a tolerance status for TNF-α and IL-6 upon LPS secondary stimulation. Interestingly, upon restimulation with Pam3CSK4, Pam3CSK4-preactivated monocytes were programmed to produce an increased amount of IL-6 compared to that

produced by nonactivated (RPMI) control cells (Fig. 3A). The anti-inflammatory-trained pattern was generally observable for TNF- α upon LPS secondary stimulation (Fig. 3A). Similarly, decreasing the amount of LPS and Flagellin during the pre-activation period of monocytes led to the opposite effect of tolerance, that is a pro-inflammatory status represented by significantly elevated levels of TNF- α and IL-6 upon restimulation (Fig. 3B and C).

We next deciphered the effect of differential engagement of cytosolic receptors. Preincubation of cells with low doses of Poly(I-C) or R848 gradually reversed the long-term tolerance effect (Fig. 4A and B), leading to a training effect upon restimulation with LPS or Pam3CSK4. Interestingly, lowering the concentrations of CpG (Fig. 4C), Tri-DAP (Fig. 4D), or MDP (Fig. 4E) did not reverse the long-term effect obtained with high inflammatory doses. Particularly, lowering the doses of CpG did not alter the “inert” status of priming, and none of the doses used triggered an enhanced or refractory immune status upon restimulation of the monocytes (Fig. 4C). However, although the enhanced proinflammatory status of the monocytes was not reversed to tolerance, the training effect obtained with the preactivation of NLRs eventually vanished by lowering the amount of Tri-DAP and MDP, resulting in normal nontolerant and nontrained cells (Fig. 4D and E). Cell viability and ligand toxicity were assessed at 24 h and at 7 days after the first stimulation for all the ligands tested, and none of the ligands used during the 24 h of primary cell stimulation enhanced LDH release

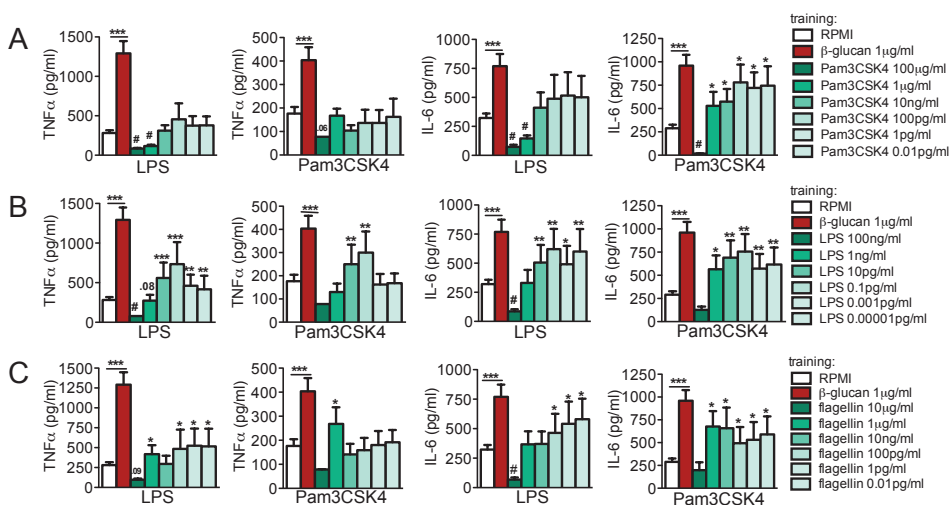


Fig. 3: Dose responses of membrane-bound receptors. The cells were preexposed for 24 h to culture medium or to different concentrations of Pam3CSK4 (100 μ g/ml to 0.01 pg/ml) (A), LPS (100 ng/ml to 10^{-5} pg/ml) (B), and Flagellin (10 μ g/ml to 0.01 pg/ml) (C). The first stimuli were washed away, and adherent monocytes were further incubated for 5 days in culture medium supplemented with 10% pooled human pool serum. At day 6, the cells were restimulated with RPMI (negative control), LPS (10 ng/ml), or Pam3CSK4 (10 μ g/ml). Depending on the concentration used, after several days, cells entered either a trained or tolerated status. The data are presented as the means \pm SEM ($n > 8$, 5 independent experiments), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$ (tolerance). Wilcoxon signed rank test was used to detect significant differences. TNF- α and IL-6 from cells stimulated with RPMI were below the detection limit.

compared to RPMI-treated cells (Supplementary Fig. S1), demonstrating that the decreased cytokine production was not due to the toxicity of the ligands used in the experiments.

Trained or tolerized cells exhibit a broad range of cell size, granulocyt, and different macrophage markers at the surface

Macroscopically, cells trained with β -glucan (1 μ g/ml), MDP (1 μ g/ml) and Flagellin (1 μ g/ml) are considerable larger than nontreated cells (Fig. 5A-D), while macrophages that entered a tolerized status were comparable in size to RPMI-treated cells (Fig. 5E and F). The phenotype of considerable larger *trained* cells is reminiscent of a previous study

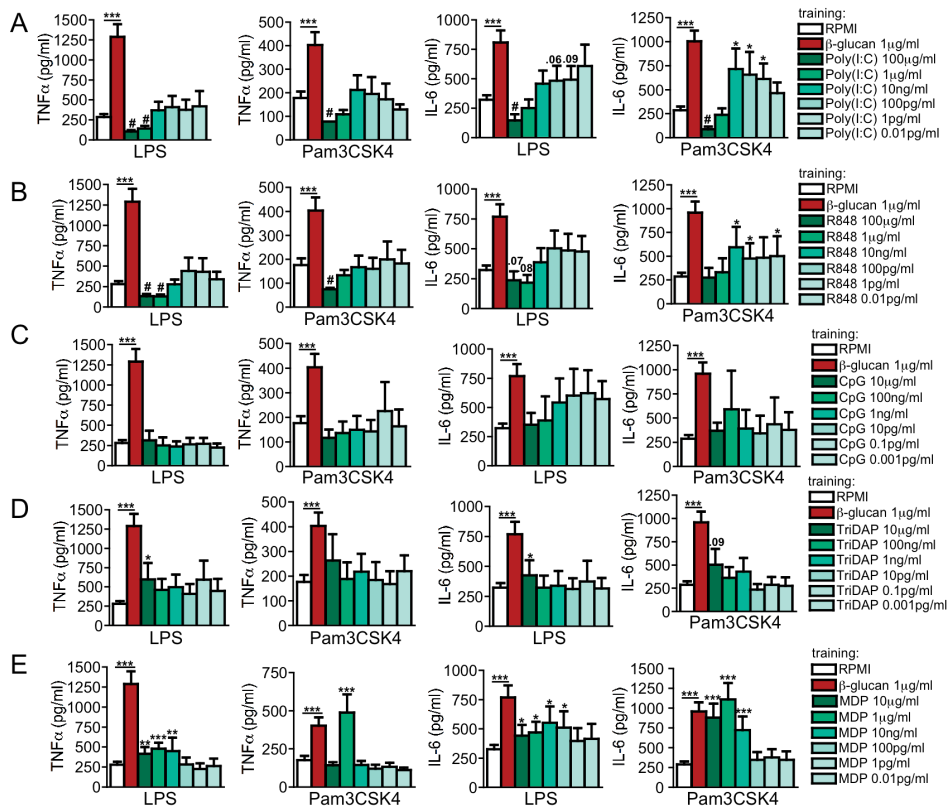


Fig. 4: Dose responses of endosomal and cytosolic receptors. The cells were preexposed for 24 h to culture medium or to different concentrations of Poly(I:C) (100 μ g/ml to 0.01 pg/ml) (A), R848 (100 μ g/ml to 0.01 pg/ml) (B), CpG (10 μ g/ml to 0.001 pg/ml) (C), Tri-DAP (10 μ g/ml to 0.001 pg/ml) (D), and MDP (10 μ g/ml to 0.01 pg/ml) (E). The first stimuli were washed away, and adherent monocytes were further incubated for 5 days in culture medium supplemented with 10% pooled human pool serum. At day 6, the cells were restimulated with RPMI (negative control), LPS (10 ng/ml), or Pam3CSK4 (10 μ g/ml). Depending on concentration used, after several days, cells entered either a trained or tolerized status. The data are presented as the means \pm SEM ($n > 8$, 5 independent experiments), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, * $p < 0.05$ (tolerance). Wilcoxon signed rank test was used to detect significant differences. TNF- α and IL-6 from cells stimulated with RPMI were below the detection limit.

by Daigneault *et al* (28), in which human monocyte-derived macrophages with serum and PMA-activated-THP1 cells via activation showed increased light scattering due to the accumulation of organelles (28). To establish the similarities and differences between long-term innate immune reprogramming by PRRs ligands from the process of PMA-induced-cell activation, we assessed the effect of two different concentrations of PMA (10 $\mu\text{g/ml}$ and 10 ng/ml) in our *in vitro* experimental model. Preincubation of monocytes with a low dose of PMA during the first 24 h resulted in an enhanced proinflammatory status, while higher concentration (10 $\mu\text{g/ml}$) induced a refractory immune status compared to the non-activated control macrophages (Supplementary Fig. S2). Hence, these results suggest the ability of PMA to induce differential activation of monocytes at the level of pro-inflammatory cytokines production, in a dose-dependent manner, similarly to TLRs ligands.

To further investigate the phenotype of *trained* versus *tolerant* cells, we assessed several surface markers on the cells treated with different PAMPs, by means of flow cytometry (Fig. 6). Adherent monocytes were trained as previously described (Fig. 1A) and after 6 days, cells were restimulated with medium alone. RPMI-trained cells are represented in Fig. 6A. All the counts were gated on CD45⁺ high (Fig. 6B and C). β -glucan was used as an inherent control; thus, based on β -glucan trained macrophages, several cell populations were identified for CD14, CD68 and DC-SIGN positive cells (Fig. 6D to F), while for CD11b⁺ only one cell population was identified (Fig. 6G). The levels of CD14⁺, CD68⁺, DC-SIGN⁺ and CD11b⁺ at the cell surface were assessed. Interestingly, based on the side and forward scatter analysis, all β -glucan-trained cells proved to be of a broad granulocytic and size range, and most of the cells positive for CD14, CD68 and CD11b (Fig. 6H to J). Similar observations were obtained with MDP and Flagellin-treated cells at a concentration that induces training (1 $\mu\text{g/ml}$) (Fig. 6H and J), while CD68⁺ and DC-SIGN⁺ were less present on the MDP and Flagellin-trained cells. Tolerant macrophages treated with Pam3CSK4 (1 $\mu\text{g/ml}$) or R848 (1 $\mu\text{g/ml}$) were significantly smaller than β -glucan-trained cells, with a broad granulocytic, and fewer were positive for CD68, CD11b or DC-SIGN, while CD14 was still present to a similar intensity as β -glucan-trained cells (Fig. 6H to K).

Trained immunity is dependent on MAP kinase-dependent pathways and histone methylation and acetylation

(MAPK) play a key role in the induction of the initial inflammatory cytokine response of the activated monocytes (29). This prompted us to investigate whether MAPK (ERK, JNK, and p38) and Syk pathways are involved in the training effects of monocytes. While β -glucan signaling induces trained immunity via the noncanonical Raf1 pathway (16), we show here that p38 and JNK are involved in the training of primary monocytes by MDP and Flagellin (Fig. 7A and B). In contrast, ERK and Syk inhibitors did not affect training by these ligands (data not shown). β -glucan and *Mycobacterium bovis* BCG-induced trained immunity is mediated by epigenetic mechanisms (16, 17). We assessed whether histone modifications by methylation or acetylation influence the observed training effects of MDP and Flagellin.

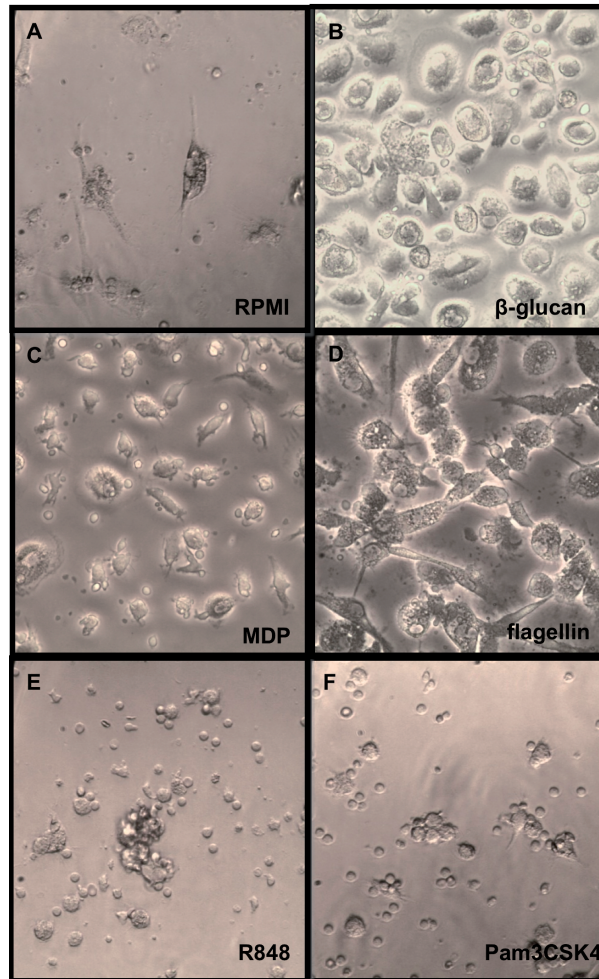


Fig. 5: Morphology of trained versus tolerized cells. The cells were preexposed for 24 h to culture medium (A) or to several pure ligands, such as β -glucan (1 μ g/ml) (B), MDP (1 μ g/ml) (C), Flagellin (1 μ g/ml) (D), R848 (1 μ g/ml) (E), and Pam3CSK4 (1 μ g/ml) (F). The first stimuli were washed away, and adherent monocytes were further incubated for 5 days in culture medium supplemented with 10% pooled human pool serum. At day 6, the cells were microscopically examined and representative pictures were chosen (x200). After several days in culture, primary monocytes differentiated into the macrophages. While trained cells appeared to be bigger and more activated (B, to D), cells that entered a tolerizing status seemed to be considerably smaller (E and F).

Inhibition of histone methyltransferases with 5'-deoxy-5'-methylthio-adenosine (MTA) or inhibition of histone acetyltransferases with epigallocatechin-3-gallate (EGCG) drastically inhibited the training of monocytes (Fig. 7C and D). In contrast, the inhibitors of histone demethylase or deacetylase enzymes did not significantly influence the training effects of MDP or Flagellin. Notably, the putative cytotoxic effects of MAPK, Syk, and epigenetic inhibitors used in the study were assessed by LDH measurements. None of the inhibitors used during

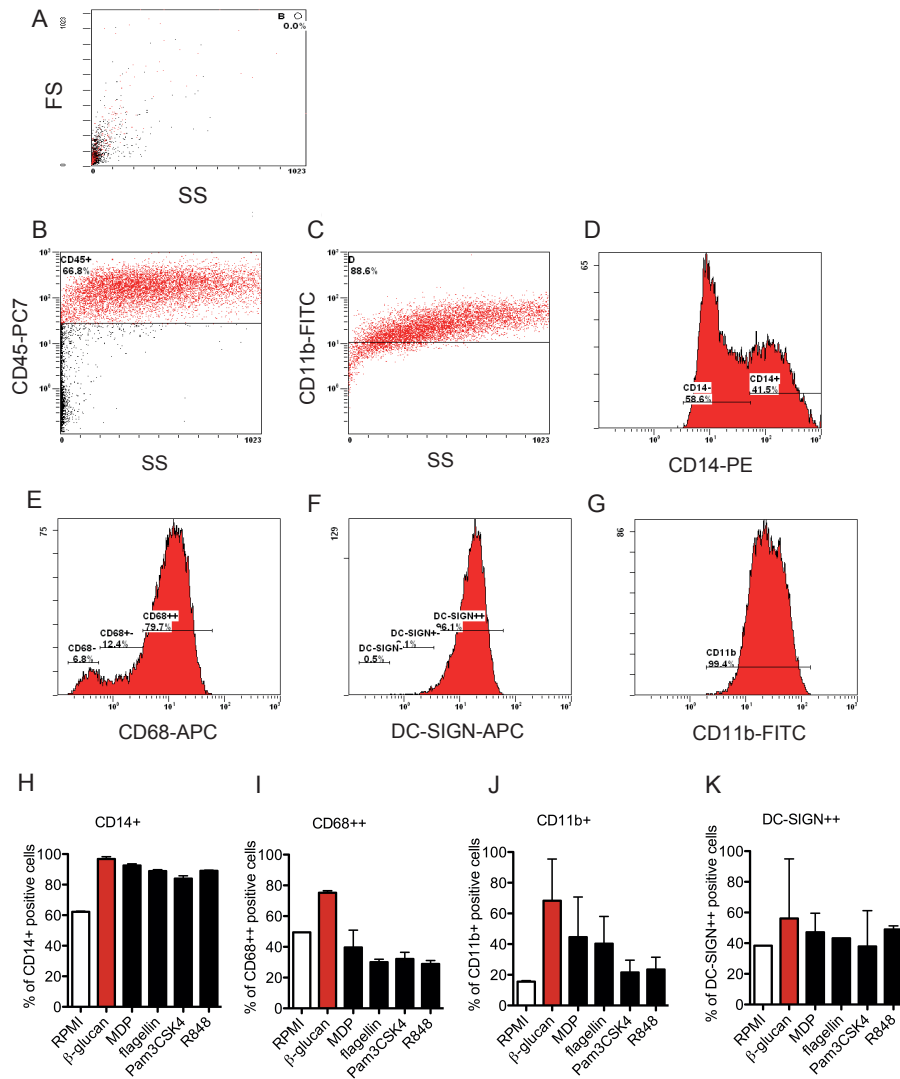


Fig. 6: Flow cytometric analysis of CD14⁺, CD68⁺, DC-SIGN⁺, and CD11b⁺ cells gated on CD45⁺ populations after prestimulation with β -glucan, MDP, Flagellin, R848, and Pam3CSK4. A total of 3 ml at 5×10^6 PBMCs was incubated for 1 h, washed 3 times with PBS, and the remaining adherent monocytes were coincubated with β -glucan (1 μ g/ml), MDP (1 μ g/ml), Flagellin (1 μ g/ml), R848 (1 μ g/ml) and Pam3CSK4 (1 μ g/ml) or with RPMI (untrained control) for 24 h. Thereafter, the cells were washed and further incubated with RPMI supplemented with 10% pooled human serum for 5 days. At day 6, the macrophages were incubated with Versene solution for 1 h at 37°C, detached from the plate, and stained extracellularly using anti-CD45 PE-Cy7, anti-CD14 PE, anti-CD68 APC, anti-DC-SIGN APC, and anti-CD11b FITC antibody. (A) The side scatter (SS) and forward scatter (FS) of the RPMI-treated cells are shown. (B and C) One representative picture of the staining and gating on CD45⁺ high β -glucan-trained cells is shown. Depending on the fluorescence intensity as well as cell size, we identified several cell populations, depicted as negative (-), positive (+), and high positive (++). Gating on different populations of CD14⁺ β -glucan-trained cells (D), CD68⁺ β -glucan-trained cells (E), DC-SIGN⁺ β -glucan-trained cells (F), and CD11b⁺ β -glucan-trained cells (G) are shown. (H to K) % of positive cells gated on CD45⁺ high. The data are presented as the means \pm SEM ($n > 4$ independent experiments).

the 24 h of primary cell stimulation enhanced LDH release compared with RPMI-treated cells (Supplementary Fig. S3), demonstrating that the molecules were not toxic to the cells.

Discussion

In the present study, we show that primary exposure to microbial ligands alters the functional fate of monocytes, which is determined by the nature and concentrations of the PRRs engaged. Engagement of NLRs (NOD1 or NOD2 receptors) induces trained immunity, a long-term enhanced immune status upon priming with high doses of MDP or Tri-DAP, which can

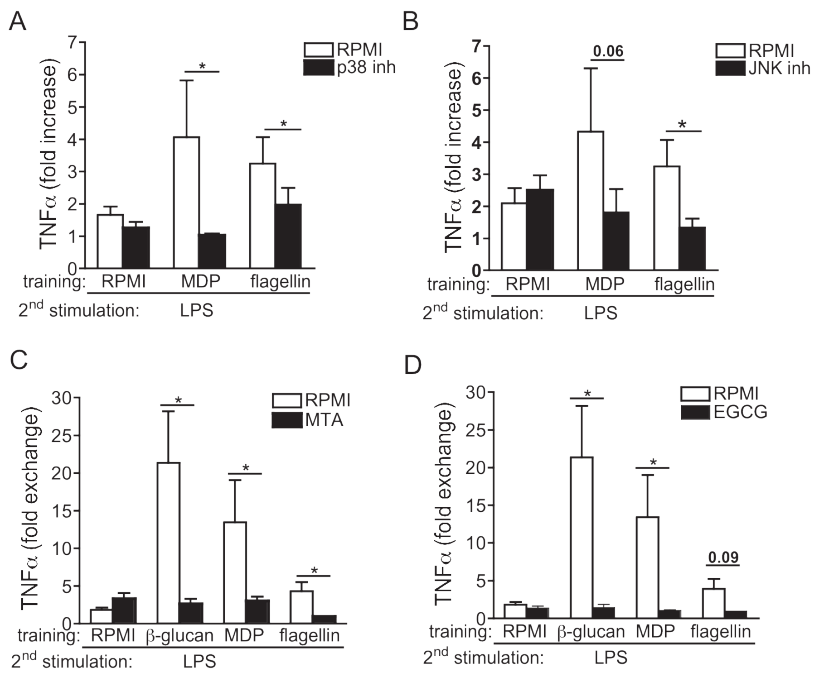


Fig. 7: (A and B) MAP kinase-dependent pathways mediate trained immunity. Adherent monocytes were preincubated for 1 h with culture medium, p38, ERK, JNK, or Syk inhibitor (inh). After this inhibition period, 100 μ l per well of either culture medium, Flagellin (1 μ g/ml) or MDP (1 μ g/ml) was added to the cell suspension, without removing the inhibitors, for 24 h. Thereafter, the cells were washed and incubated with RPMI and 10% pooled human serum. At day 6, the cells were subjected to a second stimulation with LPS (10 ng/ml) for an additional 24 h. The data are presented as means \pm SEM ($n \geq 7$, four independent experiments), * $p < 0.05$. The Wilcoxon signed-rank test was used to detect significant differences. (C and D) Blocking histone methylation and acetylation inhibits trained immunity induced by MDP and Flagellin. Before training, adherent monocytes were preincubated for 1 h with culture medium or with different epigenetic inhibitors, such as histone methyltransferase inhibitor (MTA), histone acetyltransferase inhibitor (EGCG), histone demethylase inhibitor (pargyline), and the histone deacetylase inhibitor ITF2357/9. Thereafter, 100 μ l of culture medium, β -glucan (10 μ g/ml), Flagellin (1 μ g/ml) or MDP (1 μ g/ml) was added to the cell suspension, without removing the inhibitors, and further incubated for an additional 24 h. At day 6, the macrophages were subjected to a second stimulation with LPS (10 ng/ml) for another 24 h. The data are presented as the means \pm SEM ($n = 6$, three independent experiments), * $p < 0.05$. Wilcoxon signed rank test was used to detect significant differences.

vanish with smaller amounts of ligands. The engagement of TLRs with high inflammatory doses of PRR ligands predominantly induces tolerance, with the exception of CpG, for which tolerance seems to be inherent at any dose assessed. Interestingly, low concentrations of TLR ligands not only abolished the tolerance-induced effect but also reversed it, and in fact, trained the monocytes to be maintained in a heightened proinflammatory state. Furthermore, functional reprogramming of monocytes was also associated with cell shape and cell surface marker modifications. Finally, training of monocytes by MDP and flagellin is mediated by p38 and JNK MAPK dependent pathways and depends on epigenetic histone modifications.

Earlier studies have shown that β -glucan recognition by monocytes through dectin-1 and CR3 induces trained immunity (30), which mediates protection to reinfection in a T-/B-cell-independent fashion (16). Similarly, BCG vaccination in human volunteers induces epigenetic reprogramming of monocytes and enhances secondary responses to microbial stimulation (17). In this study we report that differential engagement of PRRs and that PMA activation of monocytes might result in either an enhanced or refractory innate immune status. These findings raise an important conceptual question: what is the difference between a stimulated cell, a differentiated macrophage, and a trained immune cell? The difference can be defined at a molecular epigenetic level: the unstimulated monocyte or macrophage is characterized by the absence of cytokine production, with silent markers for both histone acetylation and methylation. During cell activation and active transcription, both histone methylation and histone acetylation are present at the promoter and enhancer levels, potentiating gene transcription. The trained cell has lost its acetylation markers and thus has lost active transcription (after an initial stimulation). However, in a trained cell (in contrast to a naïve cell), the promoters of inflammatory genes are tagged by histone methylation, which allows for an accelerated and stronger response upon restimulation. We recently described the H3K4 methylation as a property of trained monocytes (16), and this characteristic was confirmed in a landmark study by Ostuni *et al.* (31) showing the kinetic events during macrophage training. The authors proposed the name “latent enhancers” for the epigenetic units determining cell training (31). The opposing process of trained immunity is the innate immune tolerance, which has been known for more than half a century and which was recently demonstrated to be regulated by epigenetic events (21, 32, 33).

Despite the importance of innate immune training and tolerance, no systematic studies have attempted to define which microbial ligands and pattern recognition receptors induce trained immunity or tolerance. In the present study, we screened the major TLR and NLR microbial ligands for their capacity to induce either training or tolerance. Although dose dependent, we identified important bacterial ligands, such as Flagellin and MDP, which can induce trained immunity. In flagellated bacteria, Flagellin is one of the major proteins in the cell, and mammals tend to have a strong immune response to Flagellin. This molecule is recognized in mammals by Toll-like receptor 5 (34, 35). Immune responses to Flagellin have been proposed to play an important role in the pathogenesis of inflammatory bowel diseases (36, 37). From this perspective, the capacity of Flagellin to induce trained immunity and a more potent inflammatory response may have important consequences for

the pathogenesis of this disease. On the other hand, our data may lead to the conclusion that Flagellin represents an important new type of adjuvant for improving vaccination, a hypothesis that is supported by earlier studies showing that Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis* (38). Muramyl dipeptide is a component of bacterial cell wall peptidoglycan that is recognized by NOD2 (39), which is present in most bacterial species. The importance of NOD2 in protection against inflammatory bowel disease has been highlighted by the fact that particular mutations in NOD2 are associated with susceptibility to Crohn's disease (40, 41). Recently, it has been proposed that MDP functions as a mucosal adjuvant that enhances the immunogenicity of virus-like particles (42). Consequently, its capacity to induce trained immunity may represent an important advantage in these settings.

In contrast, other microbial ligands, such as the TLR9 agonist CpG, had little, if any, long-term effects on the function of monocytes. This argues that only certain ligands at certain concentrations are capable of inducing trained immunity. A particular interesting finding is that TLRs in high doses, with the exception of TLR9, consistently induced long-term tolerance to secondary stimulation with bacterial ligands. These findings are supported by earlier studies showing that the *in vitro* tolerance and crosstolerance phenomena might be induced via TLR2, TLR4, and TLR9 (43). Additionally, it has been reported that TLR7/8 engagement mediates short-term tolerance in mice both *in vitro* and *in vivo* (44, 45). Further investigation of the mechanisms mediating this effect is warranted, especially in light of the known immunosuppressive effects of viral infections (46, 47). The levels of several activation markers at the cell surface were assessed. Based on the side and forward scatter analyses, β -glucan-, MDP-, and Flagellin-trained cells are of a broad granulocytic and cell size and mainly positive for CD14 and CD11b, while cells positive for CD68 and DC-SIGN were less present. Tolerant macrophages, treated with Pam3CSK4 or R848, were significantly smaller than β -glucan-trained cells, and fewer were positive for CD68, CD11b, or DC-SIGN, while the activation marker CD14 was still present on these cells. No clear effect was seen on the expression of DC-SIGN on the macrophages. The increase or decrease in these activation markers might thus contribute to an increase or decrease, respectively, in the innate immune responses during the induction of trained immunity; nonetheless, future studies are warranted in order to prove this. In addition, one may also speculate that trained cells present different classes of activation markers than tolerized cells, depending on the subclasses of macrophages induced upon prestimulation.

An important question regarding trained immunity refers to the signaling and molecular mechanisms responsible for its induction. MAP kinases, such as p38, JNK, or ERK, are known to be crucial signaling pathways mediating the stimulation of inflammatory mediators (48, 49), with the p38MAPK α pathway being critical for normal inflammatory responses *in vivo* (50). Notably, the TRIF-dependent late-phase activation of the p38MAPK/MK2 pathway has been demonstrated to be essential for the translational control of TNF- α production (51). LPS regulates the transcript stability of TNF- α (52, 53) via activation of the p38MAPK/MK2 pathway. Considering these crucial biological functions of MAPKs, we assessed their role

in the induction of trained immunity. Our data demonstrate that JNK and p38 MAPK are central for the induction of trained immunity by Flagellin and MDP, and they might represent potential therapeutic targets for clinical situations in which trained immunity mediates a long-term inflammatory response. In contrast, ERK and Syk do not seem to be needed to elicit trained immunity. Dynamic chromatin changes across immunological pathways participate in the training mechanism induced by the preincubation of monocytes with β -glucan (16). Exposure of monocytes to β -glucans induces high levels of H3K4 trimethylation at the promoter level of inflammatory genes, which correlates with longterm increased production of proinflammatory cytokines in β -glucan-trained monocytes, a hallmark of trained immunity (16). The blockade of histone methylation or histone acetylation inhibits trained immunity; the first of these modifications has been associated with a state of transcriptional priming, while the latter is associated with actual mRNA transcription. Similar mechanisms of epigenetic memory have been demonstrated in plants during the phenomenon of systemic acquired resistance (54) and may be involved in the resistance to reinfection in invertebrates (55); both classes of organisms are devoid of adaptive immune responses. Thus, the present study demonstrates that even in the presence of adaptive immunity, the training of innate immunity is operational and serves to enhance resistance to certain types of infection.

5

A key aspect for consideration is that of the duration of trained immunity effects *in vivo* and their potential impact on hematopoietic stem cells. We recently demonstrated trained immunity effects of BCG on monocytes for up to 3 months after vaccination (17). Considering the fact that circulating monocytes have a short half-life of only days, this implies training effects of vaccination on monocyte progenitor cells, and future studies are warranted to assess this important aspect. Moreover, future studies should also assess whether the direct contact of monocytes with microbial ligands is absolutely necessary for the induction of trained immunity, or whether soluble factors, such as cytokines, may also induce similar effects in certain circumstances. In addition, one should also keep in mind that classical monocyte differentiation into macrophages also implies epigenetic changes. However, there are fundamental differences between the epigenetic elements in differentiation versus training of monocytes: in the classical differentiation, both histone methylation and acetylation epigenetic markers lead to the expression of proteins that are characteristic of a macrophage; in the training state, histone acetylation markers are lost, and the promoters of certain genes retain only the histone methylation markers characteristic of latent enhancers (31). These latent enhancers will determine the increased transcription of those genes only after cell stimulation. It can be also envisaged that in certain situations, both training and differentiation markers can be induced, with their respective functional consequences.

Our data have a number of broad implications. By inducing long-term changes in cell capacity to respond to different pathogens, the processes of trained immunity and tolerance might have important effects on the susceptibility of a host to infections. Subsequently, a logical possibility is that the microorganisms encountered by the host on a regular basis may serve to differentiate and continually renew a pool of memory-like macrophages that have enhanced responses to an infectious challenge. By identifying the receptors and signaling

pathways that determine the functional fates of monocytes and macrophages, our findings have the potential to lead to the development of new therapeutics that can harness both the potential of trained immunity to induce robust responses to enhance pathogen defenses and of immune tolerance to inhibit autoimmune phenomena.

Acknowledgements

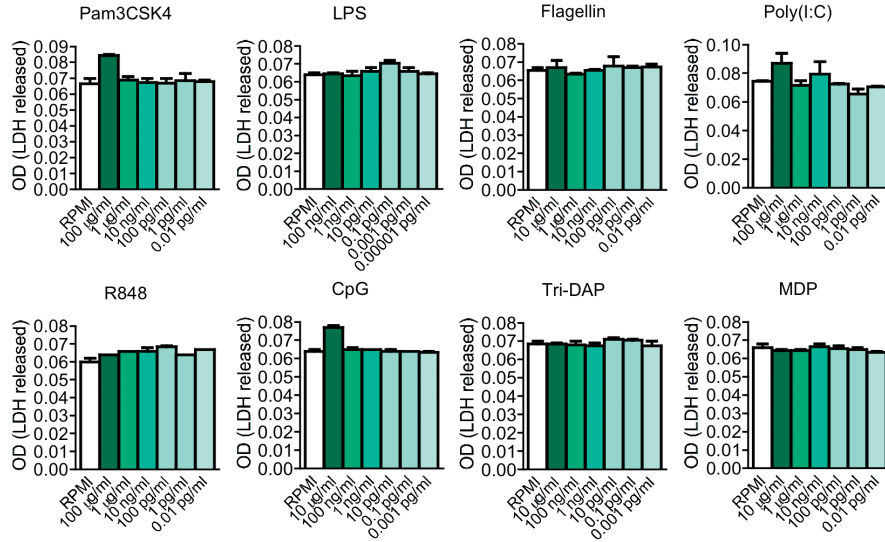
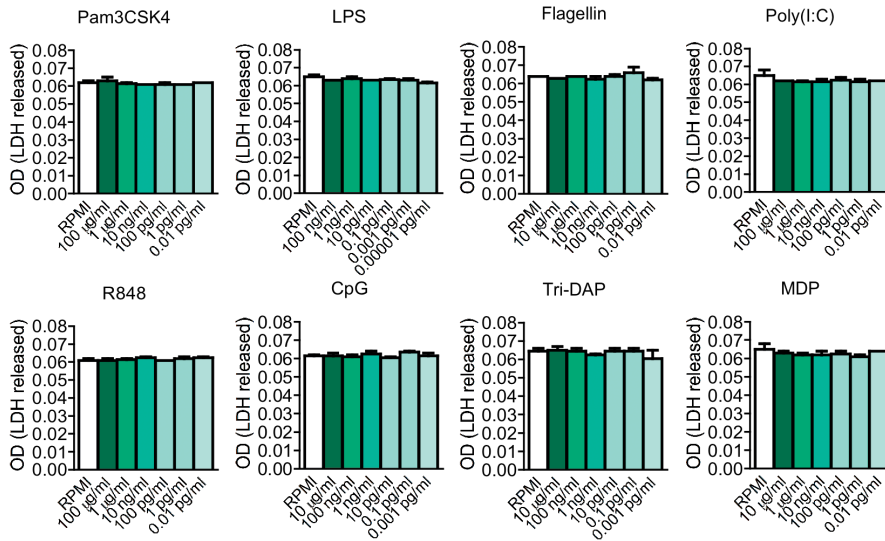
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References

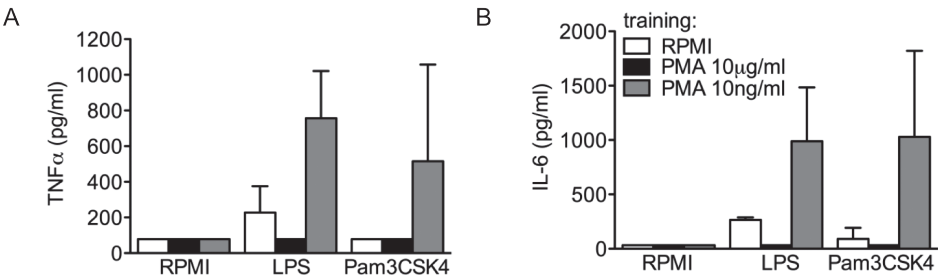
- 1 Faulhaber LM, Karp RD. A Diphasic Immune-Response against Bacteria in the American Cockroach. *Immunology*. 1992 Feb;75(2):378-81.
- 2 Rheins LA, Karp RD, Butz A. Humoral Immunity Induced in American Cockroach (*Periplaneta-Americana*). *American Zoologist*. 1978;18(3):595-.
- 3 Rheins LA, Karp RD. The Humoral Immune-Response in the American Cockroach, *Periplaneta-Americana* - Reactivity to a Defined Antigen from Honeybee Venom, Phospholipase-A2. *Developmental and Comparative Immunology*. 1984;8(4):791-801.
- 4 Sequeira T, Tavares D, AralaChaves M. Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*. *Developmental and Comparative Immunology*. 1996 Mar-Apr;20(2):97-104.
- 5 Arala-Chaves M, Sequeira T. Is there any kind of adaptive immunity in invertebrates? *Aquaculture*. 2000 Nov 20;191(1-3):247-58.
- 6 Moret Y, Siva-Jothy MT. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal Society B-Biological Sciences*. 2003 Dec 7;270(1532):2475-80.
- 7 Rheins LA, Karp RD, Butz A. Induction of Specific Humoral Immunity to Soluble-Proteins in the American Cockroach (*Periplaneta-Americana*) .I. Nature of the Primary Response. *Developmental and Comparative Immunology*. 1980;4(3):447-58.
- 8 Moret Y, Schmid-Hempel P. Entomology - Immune defence in bumble-bee offspring. *Nature*. 2001 Nov 29;414(6863):506-.
- 9 Witteveldt J, Cifuentes CC, Vlak JM, van Hulten MCW. Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. *Journal of Virology*. 2004 Feb;78(4):2057-61.
- 10 Kurtz J, Armitage SAO. Alternative adaptive immunity in invertebrates. *Trends in Immunology*. 2006 Nov;27(11):493-6.
- 11 Dong YM, Taylor HE, Dimopoulos G. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *Plos Biology*. 2006 Jul;4(7):1137-46.
- 12 Cooper MA, Elliott JM, Keyel PA, Yang LP, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 Feb 10;106(6):1915-9.
- 13 O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nature Immunology*. 2006 May;7(5):507-16.
- 14 Paust S, Senman B, von Andrian UH. Adaptive immune responses mediated by natural killer cells. *Immunological Reviews*. 2010 May;235:286-96.
- 15 Sun JC, Lanier LL. Natural killer cells remember: An evolutionary bridge between innate and adaptive immunity? *European Journal of Immunology*. 2009 Aug;39(8):2059-64.
- 16 Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* Infection Affords Protection against Reinfection via Functional Reprogramming of Monocytes. *Cell Host & Microbe*. 2012 Aug 16;12(2):223-32.
- 17 Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, et al. *Bacille Calmette-Guerin* induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 2012 Oct 23;109(43):17537-42.
- 18 Netea MG, Quintin J, van der Meer JWM. Trained Immunity: A Memory for Innate Host Defense. *Cell Host & Microbe*. 2011 May 19;9(5):355-61.
- 19 Dobrovol'skaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes and Infection*. 2002 Jul;4(9):903-14.
- 20 Fan H, Cook JA. Molecular mechanisms of endotoxin tolerance. *J Endotoxin Res*. 2004;10(2):71-84.
- 21 Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature*. 2007 Jun 21;447(7147):972-U4.
- 22 Wen H, Dou Y, Hogaboam CM, Kunkel SL. Epigenetic regulation of dendritic cell-derived interleukin-12 facilitates immunosuppression after a severe innate immune response. *Blood*. 2008 Feb 15;111(4):1797-804.
- 23 Muller A, Rice PJ, Ensley HE, Coogan PS, Kalbfleisch JH, Kelley JL, et al. Receptor binding and internalization of a water-soluble (1->3)-beta-D-glucan biologic response modifier in two monocyte macrophage cell lines. *Journal of Immunology*. 1996 May 1;156(9):3418-25.
- 24 Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: Repurification of lipopolysaccharide

- eliminates signaling through both human and murine toll-like receptor 2. *Journal of Immunology*. 2000 Jul 15;165(2):618-22.
- 25 Pinori M, Mascagni P, Mazzaferro R, Vegani B. 2007. Hydroxamic acid derivatives having anti-inflammatory action. Patent WO 97/43251 and U.S. patent 6034096.
 - 26 Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P, et al. The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. *Mol Med*. [Research Support, N.I.H., Extramural
 - 27 Research Support, Non-U.S. Gov't]. 2005 Jan-Dec;11(1-12):1-15.
 - 28 Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in Immunology*. 2009 Oct;30(10):475-87.
 - 29 Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *Plos One*. [Research Support, Non-U.S. Gov't]. 2010;5(1):e8668.
 - 30 Zhao Q, Shepherd EG, Manson ME, Nelin LD, Sorokin A, Liu YS. The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide - Attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *Journal of Biological Chemistry*. 2005 Mar 4;280(9):8101-8.
 - 31 Netea MG, Brown GD, Kullberg BJ, Gow NAR. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nature Reviews Microbiology*. 2008 Jan;6(1):67-78.
 - 32 Ostuni R, Piccolo V, Barozzi I, Polletti S, Termanini A, Bonifacio S, et al. Latent enhancers activated by stimulation in differentiated cells. *Cell*. 2013 Jan 17;152(1-2):157-71.
 - 33 ElGazzar M, Liu T, Yoza BK, McCall CE. Dynamic and selective nucleosome repositioning during endotoxin tolerance. *Journal of Biological Chemistry*. 2010 Jan 8;285(2):1259-71.
 - 34 McCall CE, El Gazzar M, Liu T, Vachharajani V, Yoza B. Epigenetics, bioenergetics, and microRNA coordinate gene-specific reprogramming during acute systemic inflammation. *J Leukoc Biol*. 2011 Sep;90(3):439-46.
 - 35 Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SLR, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature Immunology*. 2003 Dec;4(12):1247-53.
 - 36 Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001 Apr 26;410(6832):1099-103.
 - 37 Gewirtz AT. TLRs in the gut. III. Immune responses to flagellin in Crohn's disease: good, bad, or irrelevant? *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2007 Mar;292(3):G706-G10.
 - 38 Vijay-Kumar M, Gewirtz AT. Role of Flagellin in Crohn's Disease: Emblematic of the Progress and Enigmas in Understanding Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*. 2009 May;15(5):789-95.
 - 39 Honko AN, Sriranganathan N, Lees CJ, Mizel SB. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. *Infection and Immunity*. 2006 Feb;74(2):1113-20.
 - 40 Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *Journal of Biological Chemistry*. 2003 Mar 14;278(11):8869-72.
 - 41 Brant SR, Shugart YY. Inflammatory bowel disease gene hunting by linkage analysis - rationale, methodology, and present status of the field. *Inflammatory Bowel Diseases*. 2004 May;10(3):300-11.
 - 42 Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001 May 31;411(6837):599-603.
 - 43 Jackson EM, Herbst-Kralovetz MM. Intranasal Vaccination with Murabutide Enhances Humoral and Mucosal Immune Responses to a Virus-Like Particle Vaccine. *Plos One*. 2012 Jul 25;7(7).
 - 44 Dalpke AH, Lehner MD, Hartung T, Heeg K. Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology*. 2005 Oct;116(2):203-12.
 - 45 Tsukada K, Kitazawa T, Fukushima A, Okugawa S, Yanagimoto S, Tatsuno K, et al. Macrophage tolerance induced by stimulation with Toll-like receptor 7/8 ligands. *Immunology Letters*. 2007 Jul 31;111(1):51-6.
 - 46 Bourquin C, Hotz C, Noerenberg D, Voelkl A, Heidegger S, Roetzer LC, et al. Systemic Cancer Therapy with a Small Molecule Agonist of Toll-like

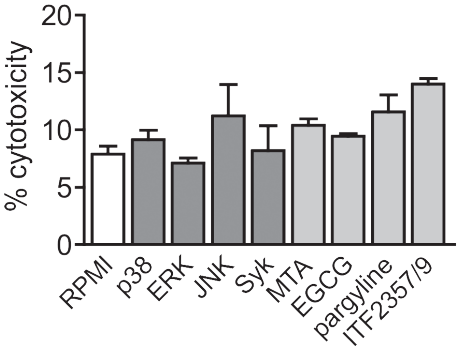
- Receptor 7 Can Be Improved by Circumventing TLR Tolerance. *Cancer Research*. 2011 Aug 1;71(15):5123-33.
- 47 Rouse BT, Horohov DW. Immunosuppression in Viral-Infections. *Reviews of Infectious Diseases*. 1986 Nov-Dec;8(6):850-73.
- 48 Brenner T, Rosenhagen C, Hornig I, Schmidt K, Lichtenstern C, Mieth M, et al. Viral Infections in Septic Shock (VISS-Trial)-Crosslinks Between Inflammation and Immunosuppression. *Journal of Surgical Research*. 2012 Aug;176(2):571-82.
- 49 Shepherd EG, Zhao Q, Welty SE, Hansen TN, Smith CV, Liu YS. The function of mitogen-activated protein kinase phosphatase-1 in peptidoglycan-stimulated macrophages. *Journal of Biological Chemistry*. 2004 Dec 24;279(52):54023-31.
- 50 Liu YS, Shepherd EG, Nelin LD. MAPK phosphatases - regulating the immune response. *Nature Reviews Immunology*. 2007 Mar;7(3):202-12.
- 51 Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. *Biochemical Journal*. 2010 Aug 1;429:403-17.
- 52 Gais P, Tiedje C, Altmayr F, Gaestel M, Weighardt H, Holzmann B. TRIF Signaling Stimulates Translation of TNF-alpha mRNA via Prolonged Activation of MK2. *Journal of Immunology*. 2010 May 15;184(10):5842-8.
- 53 Anderson P. Post-transcriptional control of cytokine production. *Nature Immunology*. 2008 Apr;9(4):353-9.
- 54 Carballo E, Cao HP, Lai WS, Kennington EA, Campbell D, Blackshear PJ. Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. *Journal of Biological Chemistry*. 2001 Nov 9;276(45):42580-7.
- 55 Durrant WE, Dong X. Systemic acquired resistance. *Annual Review of Phytopathology*. 2004;42:185-209.
- 56 Kurtz J. Specific memory within innate immune systems. *Trends in Immunology*. 2005 Apr;26(4):186-92.

A 24 h after the training**B 7 days after the training**

Supplementary Fig. 1: LDH cytotoxicity assay. Adherent monocytes were cocultured for 24 h with either culture medium or with different microbial ligands. Thereafter, supernatants were collected (A) and cells were further cultured in RPMI supplemented with serum for another 6 days. At day 7 supernatants were once again collected (B) and subjected to a cytotoxicity assay, conform to manufacturer's instructions. All the ligands, independently on the concentration, were proven to be non-toxic to the cells. Data are presented as mean \pm SEM ($n = 2$).



Supplementary Fig. 2: The influence of PMA on prestimulation of primary monocytes. Cells were pre-exposed for 24 h to culture medium or to PMA at two different concentrations: 10 μg/ml and 10 ng/ml. The first stimuli were washed away and adherent monocytes were further incubated for 5 days in culture medium supplemented with 10% pooled human serum. During the 6 days of incubation period, monocytes differentiate into macrophages. Cells were further subjected to a second stimulation with LPS (10 ng/ml) or Pam3CSK4 (10 μg/ml) for an additional 24 h. TNF-α (A) and IL-6 (B) were assessed in the cells culture supernatants. Data are presented as mean ± SEM (n = 3).



Supplementary Fig. 3: LDH cytotoxicity assay. Adherent monocytes were cocultured for 24 h with either culture medium or with p38-, ERK-, JNK-, Syk- and epigenetic inhibitors (MTA, EGCG, pargyline and ITF2357/9). Thereafter supernatants, as well as cells lysates, were collected and subjected to a cytotoxicity assay, conform to manufacturer's instructions. MAPK-, Syk- as well as epigenetic inhibitors were proven to be non-toxic to the cells. Data are presented as mean ± SEM (n = 2).

6

**Fungal chitin induces trained immunity
in human monocytes during cross-talk
with *Saccharomyces cerevisiae***

Lisa Rizzetto,* Daniela C. Ifrim,* Noemi Tocci, Shih-Chin Cheng, Jessica Quintin,
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Submitted for Publication.

* Equal contribution

Abstract

The immune system is essential to maintain the mutualistic homeostatic interaction between the host and its micro- and mycobiota. Living as a commensal on human skin and being a passenger in the digestive tract, *Saccharomyces cerevisiae* could potentially modulate the host immunity and significantly shape the immune response. We observed that diverse *S. cerevisiae* strains induce trained immunity in monocytes through a strain-dependent manner leading to enhanced cytokine production upon secondary stimulation with TLR ligands and bacterial commensals. These features are reflected by the differences in the pro-inflammatory properties dependent on the origin of the strains, which may be potentially related to the different adaptation to the environment from which they were isolated. We also established that in addition to β -glucan, *S. cerevisiae* chitin drives induction of trained immunity at several levels: increased cytokine production, but also direct antibacterial activity. Differential chitin content accounts for the differences in training properties observed among strains, with the clinical strains being the most effective. This study reveals how commensal and passenger microorganisms could be important in promoting health and preventing mucosal diseases by modulating host defense and regulating the microbiota. Dietary supplementation of specific probiotic microorganisms may be a viable strategy to train a healthy immune system.

Keywords: trained immunity, *S. cerevisiae*, strain diversity, inflammation, TLR ligands

Introduction

In several higher organisms, including plants, a central mechanism for efficient protection from infection is defense priming, the preconditioning of immunity induced by microbial colonization after germination or birth (1,2), supporting the relevance of innate immune memory (3). This property of host defense is also referred as ‘trained immunity’, defined as an increased responsiveness of the innate immune system to secondary stimuli after an initial encounter with training microorganisms (4,5).

Similarly to root symbioses, human microbiota functionally contributes to the nutrition and protection of the host, significantly shaping mammalian immunity both at the mucosal surface of the host and systemically (6–9). Recent studies have shown that fungal microbiota, or mycobiota, is an important player in the host-microbes interaction, and that its effects are integrated with those of the dominant bacterial component (10–12). The complex interaction between microbiota, mycobiota and the immune system is critical for establishing a balance between immunity and tissue health. A recent publication (9) added another fundamental contribution to the role of skin microbiota in activating and educating host immunity, shedding new light on the interplay between immune systems and microbiota in shaping each other. It has been earlier demonstrated that *C. albicans* triggers differential immune signaling upon interaction with either inflammatory or tolerogenic dendritic cells (6) and, similarly to what occurred for *Aspergillus fumigatus* (8) and harmless fungi such as *Saccharomyces cerevisiae* (13), the type and intensity of immune reactivity is strain dependent (7). Moreover, fungi modulate responses towards other microorganisms such as intestinal and skin bacteria (14,15).

Candida spp. are not the only fungal colonizer of humans. *Saccharomyces cerevisiae* has been earlier demonstrated to be a constant colonizer of skin (16) and human intestinal tract (17), and a recent study has shown that it may even surpass *Candida* species in some human populations (18). Even if it is a harmless fungus, *S. cerevisiae* shares with *Candida* many of the same cell wall structures, often leading to similar effects on host defense. In this work, we assessed the capacity of various *S. cerevisiae* strains to train human myeloid cells to react stronger towards other microorganisms such as bacteria or *Candida*. *S. cerevisiae* can efficiently induce trained immunity in human monocytes in a strain and strain origin-dependent manner, leading to enhanced cytokine production *in vitro*. This effect was only partially dependent on β -glucan and mannan, and was mainly driven by chitin, explaining the major training properties observed upon challenge with strains presenting a chitin-rich cell wall, i.e. Crohn’s disease (CD) isolates. The differences in the stimulatory capacities of *Saccharomyces* depending on the strain and the context from which they were isolated underlines the need to understand the interaction between mycobiota and the host, in order to identify the boundaries between friend and foe, and between health and disease.

Methods

Blood samples. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats provided by the Sanquin Bloodbank in Nijmegen, The Netherlands or at Careggi Hospital in Florence, after obtaining informed consent (ethical approval document n. 87/10). The study was designed in conformity with the international recommendation (Dir. EU 2001/20/EC) and its Italian counterpart (DM 15 Luglio 1997; D.Lvo 211/2003; D.L.vo 200/2007) for clinical trial and following the Declaration of Helsinki, to assure protection and care of subjects involved. Dectin-1-deficient cells were isolated from patients homozygous for the stop mutation Y238X (39).

Reagents. Ficoll-Paque (GE Healthcare) was used to isolate PBMCs by differential centrifugation. RPMI 1640 Dutch Modification (RPMI) (Sigma-Aldrich), supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate (Life Technologies, Nieuwekerk, The Netherlands) was used as culture medium. Monocytes were isolated using magnetic CD14 positive selection (Miltenyi Biotec). In a challenge experiment, IL-4 and GM-CSF (both from Gentaur, Belgium) were added to induce monocyte differentiation into dendritic cells. *C. albicans* and *S. cerevisiae* β -glucan and mannan were isolated and purified as described previously (40,41). Pam₃Cys₄ and LPS (*Escherichia coli* serotype 055:B5) were purchased from Sigma-Aldrich, with an additional purification step for LPS (42). Only preparation with > 98% purity were used in the experiments.

Microorganisms. *S. cerevisiae* strains, previously isolated from different environmental sources, were cultured in complete medium (YPD, 2% yeast extract, 1% peptone, 2% glucose) for 18 h, then collected. *C. albicans* ATCC MYA-3573 (UC 820), was grown overnight to yeast cells in Sabouraud broth at 37°C. *Staphylococcus aureus* and *Escherichia coli* were grown overnight in Tryptic Soy Broth (TSB, Difco) at 37°C. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in culture medium (RPMI; ICN Biomedicals, Aurora, OH). For trained immunity assays, *S. cerevisiae* yeasts and *Candida* were heat-killed for 30 min at 95°C and resuspended in culture medium to a cell inoculum size of 10⁶ cells/ml.

Cell wall characterization and chitin purification. Cell wall characterization has been performed as described by Ferreira et al. (2006) (43) with modifications. Cells were cultured in YPD liquid medium at 28°C and collected at late exponential phase. The cells were washed once with distilled water and three times with 0.1 M Tris-HCl buffer (pH 8.5) containing 1mM phenyl sulphonyl fluoride and disrupted in the same buffer with 0,5 mm-diameter glass beads by serial cycles of vortexing (30s at maximum speed) and ice cooling (20s). Cell walls were harvested by centrifugation for 30 minute at 4200 g and dried in a speedvac concentrator. To extract chitin, cell wall dried biomass was subjected to alkaline extraction, followed by acid extraction according to the protocol described by Ferreira et al. (2006) (43). Chitin was finally obtained by dialysis of the extract and lyophilized to determine the dry weight. Only preparation with > 98% purity were used in the experiments.

Cell preparation. The PBMC fraction was obtained by density centrifugation of diluted blood (1:1 ratio between blood and pyrogen-free saline) over Ficoll-Paque. PBMCs were washed twice

in saline and resuspended in culture medium. Monocytes were isolated from low density PBMCs by magnetic enrichment with anti-CD14 beads (Miltenyi Biotec). In a challenge experiment, monocytes were cultured in the presence of GM-CSF (800 U/ml) and recombinant IL-4 (1000 U/ml) for 6 days to allow DC differentiation (44). DC activation was induced by *S. cerevisiae* strains. A serial dilution of live yeast preparations was added to the moDCs at different stimulatory-to-DC ratios. For confirmation experiments, PBMCs from the same healthy subjects were used; stimulation was performed as in DC challenge experiments. Depending on the experiment, supernatants were collected after 24 h or 5 d, and stored at -20°C until the assays.

In-vitro induction of trained immunity. Monocytes (10^6 cells/ml) were added in a volume of 100 μ l/well in flat-bottom 96-well plates (Greiner, Nurnberg, Germany). Cells were incubated with one of the first stimuli for 24 h, rested for 5 days and re-incubated for another 24 h with one of the second stimuli. As priming stimuli we used 1×10^4 cells/ml of the different *S. cerevisiae* strains, *S. cerevisiae*- or *Candida*-derived β -glucan (10 μ g/ml), *S. cerevisiae*-derived mannan (10 μ g/ml) or chitin (10 μ g/ml). After 24 h, cells were washed to remove all stimuli, and they were allowed to rest for 5 days before being exposed to a second stimulation with various stimuli. The second stimuli were one of the following: LPS (10 ng/ml), Pam₃Cys₄ (10 μ g/ml), *Staphylococcus aureus* (1×10^7 /ml), or heat killed *C. albicans* (1×10^6 /ml) in a final total volume of 200 μ l. To investigate whether priming could be blocked, monocytes were incubated for 1 h with dectin-1/CLEC7A Ab (dectin-1 antagonist; 10 μ g/ml) or with the isotype control (10 μ g/ml), before priming. After this, cells were treated with either RPMI or *S. cerevisiae* derived β -glucan (10 μ g/ml) and incubated for 24 h, followed by a second stimulation with LPS (10 ng/ml) for an additional 24 h. Cytokines were measured after the second stimulation.

Cytokine assay. TNF- α and IL-6 levels on monocytes were determined by enzyme-linked immunosorbent assays on 24 h monocyte cell culture supernatants according to manufacturer's instructions (R&D Systems). For the other experiments, at the indicated times, supernatants from human cell cultures were collected and cytokine detection was performed using the Milliplex® MAP human cytokine/chemokine kit (Millipore), according to the manufacturer's instructions.

Microorganism survival following uptake by primed monocytes. Monocytes (10^6 cells/ml) were added in a volume of 100 μ l/well in flat-bottom 96-well plates (Greiner, Nurnberg, Germany). Cells were incubated with one of the first stimuli for 24 h, rested for 5 days and re-incubated for another 24 h with one of the second stimuli. As a priming stimulus, we used 1×10^4 cells/ml of YP4 *S. cerevisiae* strain, *S. cerevisiae* derived β -glucan (10 μ g/ml), *S. cerevisiae* derived mannan (10 μ g/ml) or YP4 derived chitin (10 μ g/ml). After 24 h, cells were washed to remove all stimuli, and they were allowed to rest for 5 days before being exposed to a second stimulation with various stimuli. The second stimuli were *E. coli* (1×10^7 /ml), *S. aureus* (1×10^7 /ml), or *C. albicans* (1×10^6 /ml) in a final total volume of 200 μ l. After 6 h of stimulation, monocytes were collected, washed three times with PBS, treated with zymolyase (2 mg/ml) or lysozyme (10 mg/ml), and washed twice; cells lysated with a hypotonic solution (0.05% KCl) to release intracellular microorganisms were plated on solid complete medium (YPD for yeast cells, TSB for bacterial cells). Survival of microorganisms after uptake are

reported as the percentage of CFUs after 1 day (bacteria) or 3 days (fungus) relative to the total number of cells growing in the absence of monocytes exposure. Control experiments were carried out to verify that the hypotonic solution was not toxic to bacteria and yeasts.

***In vitro* phagocytosis assay.** Primed monocytes (as above) will be seeded into poly-L-lysine slides and incubated for 30 min at 37°C and 5% CO₂ and then infected with *E. coli* or *C. albicans*. For *C. albicans* internalization evaluation, after PBS washing and medium replacement, cells were infected with Oregon green 488 (Molecular Probes, Life Technologies) prelabelled *Candida* cells (1X10⁶ cells/mL in complete RPMI) and further incubated for 1.5 h. Thereafter, calcofluor white (Sigma-Aldrich) was added (20 µL per well) 15 min before each end point. Cells were washed with PBS to remove nonadherent yeasts and fixed with cold ethanol for 30min at -20°C and labeled in red with PHK26 (Sigma-Aldrich). Calcofluor white staining of fungi allows one to discriminate between adherent (Calcofluor white accessible) and phagocytized (i.e. internalized, Calcofluor white) yeast cells. When visualized by epifluorescence microscopy, all the yeast cells appeared as green (independently of the localization), while the not-internalized yeast cells were blue stained. Finally, merging of Oregon green 488 and Calcofluor white images allowed one to definitively exclude the extracellular yeast cells. By contrast, *E. coli* were labeled using Oregon green (Molecular Probes, Life Technologies) only without distinguishing between internalized or not internalized bacteria. Cells were stained NucBlue Live Cell Stain Ready Probes reagent (Molecular Probes). A minimum of 200 monocytes per group were scored and any cell containing one or more particles was counted as phagocytic.

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Phagolysosome acidification assay. To visualize the acid yeast-containing vesicles, chamber slides were prepared as in Phagocytosis assay; then, primed monocytes were exposed to the acidotropic dye LysoTracker DND-99 (75 nM) and infected as described above. Afterwards the samples were fixed with cold ethanol and immediately examined by fluorescence microscopy. Acidification control groups consisted of uninfected cells. A minimum of 200 yeast/bacteria-containing phagosomes was scored; the percent of acid phagolysosomes was evaluated as the ratio between the number of LysoTracker-labelled phagosomes and the total number of yeast-containing phagosomes.

Statistical analysis. The paired Wilcoxon test was used to compare differences between the effect of a particular stimulus and the RPMI medium control. The level of significance was set at $p < 0.05$.

Results

S. cerevisiae isolates train differently the cytokine responses towards TLR ligands and fungi

As *C. albicans* enhanced the cytokines response towards TLR ligands and colonizing bacteria (14), we tested whether *S. cerevisiae* was also able to induce trained immunity in monocytes. To investigate this effect, monocytes from healthy donors were first exposed to low doses of *S.*

cerevisiae (Fig. 1A) and *C. albicans* for 24 hr (Fig. 1B). After that, the initial stimulus was removed and the cells were washed. After an additional 5-days resting period, cells were restimulated with pure components of the bacterial cell wall or with *C. albicans* (Figs. 1A and B). Exposure of monocytes to a laboratory strain of *S. cerevisiae* yeast potentiated the production of TNF- α induced by a second bacterial stimulus such as LPS, the lipopeptide analog Pam₃Cys₄, or by the fungal pathogen *C. albicans* (Fig. 1A) to the same extent as *C. albicans* (Fig. 1B).

Considering the variability in stimulating pro- and anti-inflammatory cytokines of fungal strains (8,13), we probed the ability of a set of *S. cerevisiae* strains to induce trained immunity towards different microbial ligands such as *E. coli* LPS (Fig. 2A) and Pam₃Cys₄ (Fig. 2B). This set comprises: 2 laboratory strains (SK1 (19) and BY4741 (20) used as reference strains in previous studies (21)), 9 clinical isolates (13) - 8 from CD patients, one from healthy subject -, 4 wild strains from Barriada wine region of Portugal, and 4 grape isolates from Tuscan vineyards (22). The results showed that different *S. cerevisiae* strains differentially train TNF- α production in response to both TLR ligands, in a similar or even higher extent than *C. albicans* (14). In contrast to the clinical and Tuscan grape isolates, laboratory and Barriada wine (wild) strains showed little

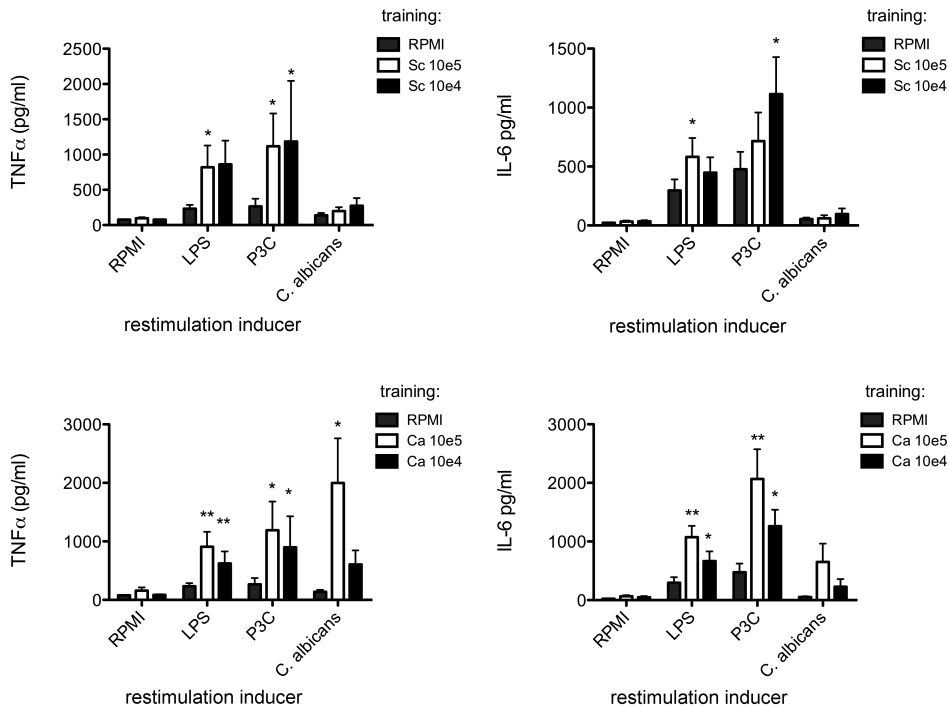


Figure 1. *In vitro* training of monocytes with *S. cerevisiae*. Monocytes were trained with *S. cerevisiae* (A) or *C. albicans* (B) (priming stimulus) and after 24 h washed to remove the stimulus. TNF- α and IL-6 were measured in supernatants after bacterial cell wall pure components or *C. albicans* second stimulation (n = 8; four independent experiments). Bars indicate mean + SD (n = 10, four independent experiments). *p < 0.05 trained cells versus RPMI-stimulated, Wilcoxon nonparametric test for two related samples. P3C, Pam₃Cys₄.

ability to train the monocytes response to Pam₃Cys₄ (Figs. 2A and B). All strains were able to modulate TNF- α and IL-6 production upon challenge with LPS, albeit to different extents. The clinical YP4 isolate showed the greatest potential to train cytokine production (Figs. 2A and B).

Since one of human commensal microorganisms is *C. albicans* itself, we tested whether *S. cerevisiae* isolates from different environmental niches train cytokine production in response to *C. albicans* stimulation. As shown in Fig. 2C, while the healthy human isolate Y13EU was not able to modulate TNF- α and IL-6 production, clinical and wild isolates enhance monocyte response to *C. albicans* with different extents.

β -glucans from *S. cerevisiae* mediate training partially through Dectin-1

As in the case of *C. albicans*, the outer layer of the cell wall of *S. cerevisiae* is enriched with mannoproteins, whereas the inner layer is composed of chitin and β -glucan. Both mannosylated proteins, phospholipomannan and β -glucan are known inducers of proinflammatory cytokines through C-type lectin receptors (23). Therefore, we assessed whether purified β -glucan from *S. cerevisiae* (Sc) was able to exert a training effect similar to that obtained with *S. cerevisiae* whole organism or *C. albicans* (Ca)-derived β -glucan (14). *S. cerevisiae*-derived β -glucan trained the monocytes and led to an increased amount of TNF- α induced by the secondary stimuli (Fig. 3A). When we blocked the β -glucan receptor dectin-1 with an anti-dectin-1 antibody, β -glucan training effect was considerably reduced (~50%), but not abolished, whereas the isotype control antibody had no effect (Fig. 3B). To note that, the incomplete suppression of the training activity could be due to the little traces of mannan and chitin in the β -glucan preparation. We completed the assessment of the role of dectin-1 by using primary monocytes isolated from four dectin-1 deficient volunteers. In dectin-1-deficient cells β -glucan training was partially abrogated, validating the role of the dectin-1 receptor in mediating the β -glucan-dependent trained immunity (Fig. 3C).

However, although these results show that β -glucan exerts a strong training effect, this mechanism does not account alone for the effect induced by the whole microorganism as revealed by using dectin-1 deficient cells (Fig. 4A). Thus, we assessed the priming potential of *S. cerevisiae*-derived mannan alone and in combination with β -glucan (Fig. 4B). As shown, mannan alone could induce an increase of TNF- α production in response to a secondary infection, but the effect is merely additive to that of β -glucan, revealing that mannan is able to potentiate the training effect shown by β -glucan. This seems to be in contrast to what is observed in *C. albicans*, but in line with the differential branching of *S. cerevisiae* and *Candida* fungal mannan, which determines different immune reactivity (21, 24).

Fungal chitin drives/potentiates the immune training and potentiate intracellular killing of fungi and bacteria

Differential mannan branching only partially clarifies the different training properties shown by the diverse *S. cerevisiae* isolates. The isolates from patients we tested showing the most significant ability to modulate cytokine production to a secondary bacterial stimulation (Figs.

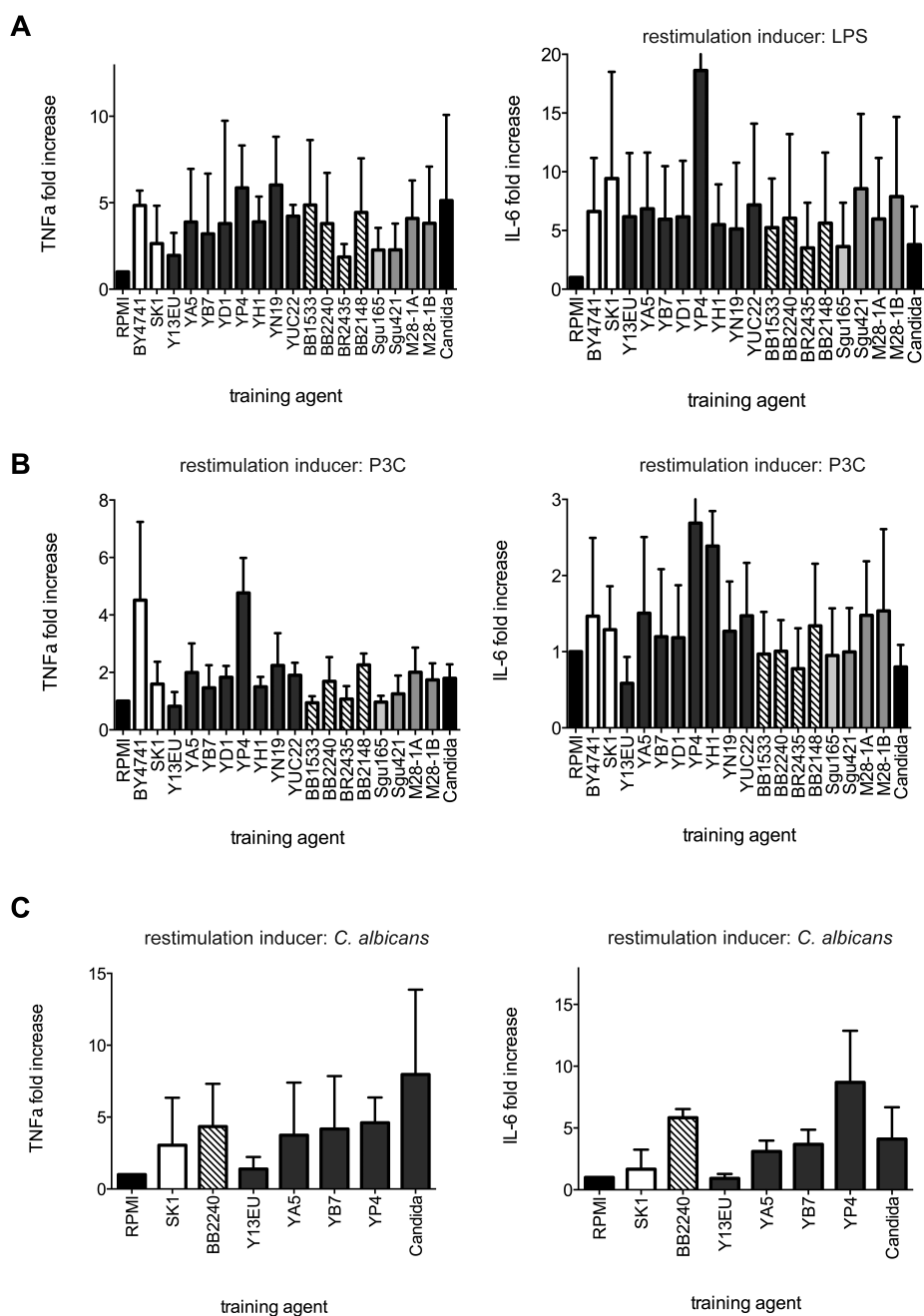


Figure 2. *S. cerevisiae* strains enhance differently the cytokine responses of cells restimulated with TLR ligands. **A.** *In vitro* training of monocytes with different strains of *S. cerevisiae* towards LPS (A), Pam3Cys4 (B) or *C. albicans* (C) stimulation. White bars: laboratory strains; dark grey: clinical strains; light grey: Tuscan wine isolates; dashed: Barriada wild isolates. Bars indicate mean + SD (N=6). *p<0.05 trained cells versus RPMI-stimulated, Wilcoxon nonparametric test for two related samples. P3C, Pam₃Cys₄.

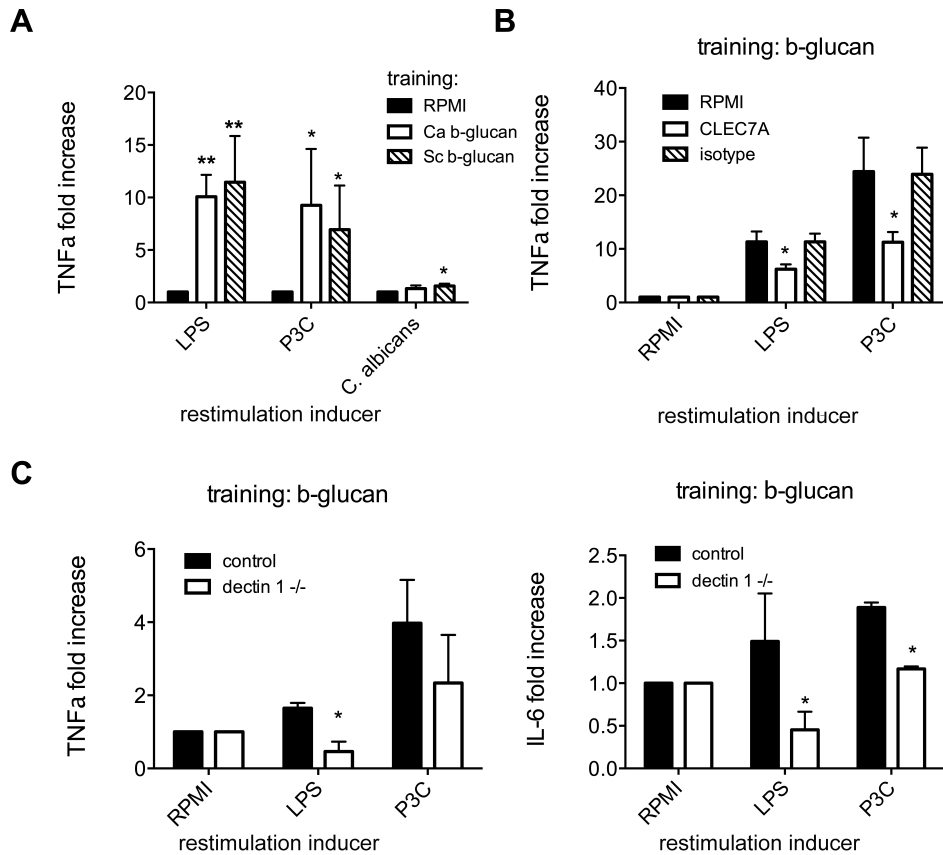


Figure 3. β -glucans are essential, but not sufficient, in enhancing cytokine production mediated by *S. cerevisiae*. A. Monocytes were incubated with RPMI or *S. cerevisiae* β -glucan (10 μ g/ml), period after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were reincubated with pure ligands or heat-killed bacteria. B. Monocytes were incubated with RPMI, CLEC7A Ab, and the isotype control (10 μ g/ml). After 1 hr of incubation, cells were treated either with RPMI or β -glucan (10 μ g/ml) and, after 5 days of resting, restimulated with LPS or Pam3Cys4. C. Monocytes from 4 healthy and 4 dectin-1-deficient subjects were incubated with RPMI and β -glucan (10 μ g/ml) for 24 h, followed by a second stimulation with TLR ligands for an additional 24 h after 5 days of resting. For all the experiments, TNF- α and IL-6 were measured in supernatants after the second stimulation. Data are presented as fold increase with respect to not-trained (RPMI-stimulated) cells. For (A), $n = 10$; for (B), (C), $n = 4$. Bars indicate mean \pm SD. * $p < 0.05$, ** $p < 0.01$ versus RPMI-stimulated cells, Wilcoxon nonparametric test for two related samples. P3C, Pam₃Cys₄.

2C and 3A) were isolated from pediatric CD patients (13). Next, we investigated whether the strains isolated from different environments induce different inflammatory properties, which might explain the differential training. Therefore, in parallel to the current investigation, we screened the immune reactivity of monocyte-derived dendritic cells to an enlarged set of *S. cerevisiae* strains. This investigation showed a wide diversity in the immune-modulatory profiles among the different strains isolated from diverse ecological niches, quantified directly by cytokine production capacity comparison (S1 Fig.). In general, the different isolates showed

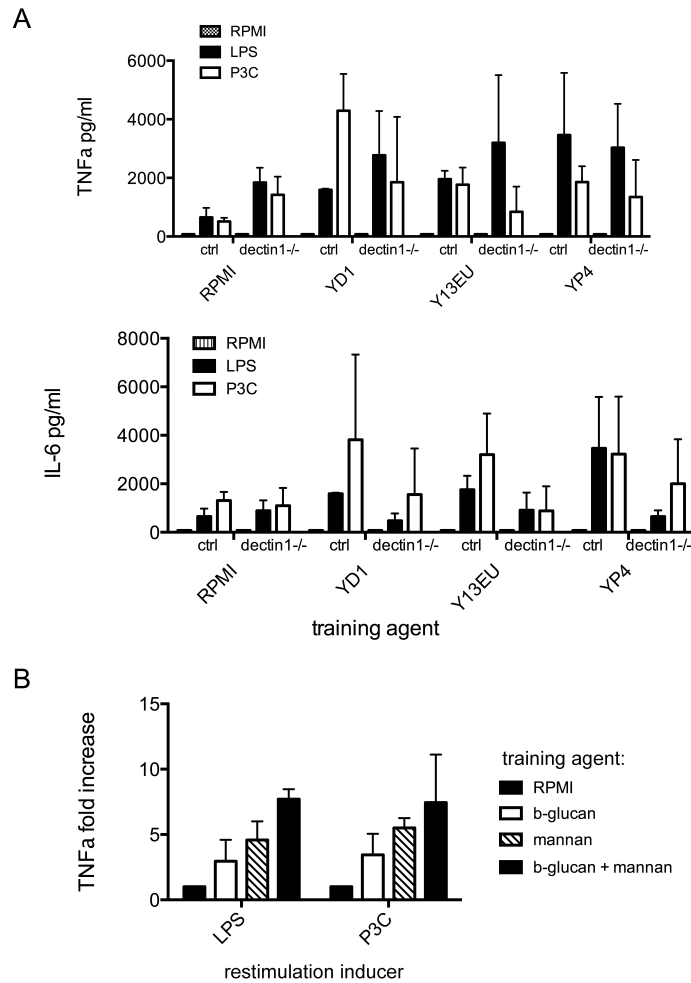


Figure 4. Training with *S. cerevisiae* strains isolated from Crohn's disease patients is partially dectin-1-dependent, and mannans are important to potentiate the cytokine modulation. **A.** Healthy and dectin-1-deficient monocytes 4 were incubated with RPMI and clinical *S. cerevisiae* isolates for 24 h, after which the cells were washed to remove the stimulus. This was followed by a second stimulation with TLR ligands for an additional 24 h after 5 days of resting. TNF-α and IL-6 were measured in supernatants. **B.** Monocytes were exposed to RPMI, β-glucan (10 μg/ml), *S. cerevisiae* derived mannan (10 μg/ml) or a combination of both for 24 h, after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated with pure ligands. TNF-α was measured in supernatants. (A, B), Bars indicate mean ± SD (n=4). *p < 0.05, LPS trained dectin1^{-/-} cells versus LPS trained control cells. *p < 0.05, P3C trained dectin1^{-/-} cells versus P3C trained control cells. Wilcoxon nonparametric test for two related samples. LPS, lipopolysaccharide; P3C, Pam₃Cys₄.

an immune-based diversity that seemed to correlate with their origin, as shown by clustering of the 6 cytokine profiles (S2A Fig.) and principal component analysis (S2B Fig.).

The interesting cytokines profile observed was confirmed using PBMCs for stimulation with live high- and low-inflammatory strains (S3 Fig.). The different isolates showed

diverse ability to promote IL-17 and IFN- γ production, and their inflammatory role was counterbalanced by different extents by IL-10 induction. We speculate that differences in the cell wall composition of the isolates tested influenced their ability to induce and train cytokine production, and could be the result of the different environmental selective forces. Thus, we investigated the cell wall composition of 4 strains with different ecological niches: the laboratory SK1 strain, the environmental BB2240 strain, the CD's YP4 isolate and the healthy isolated Y13EU. While β -glucan content did not differ among the isolates, chitin was almost 10-fold more prevalent in the CD isolate with respect to the laboratory and the environmental strains (Fig. 5A). We isolated chitin from those strains and assessed the immune training properties of this fungal cell wall component. Chitin was able to induce a potent immune response in terms of IL-6 and TNF- α induction (Fig. 5B).

We thereafter compared the ability of whole *S. cerevisiae* cell and its cell wall component to induce trained immunity for killing both fungi and bacteria. In this set of experiments, monocytes trained as previously described were exposed for 6 h to live fungus *C. albicans*, to the Gram-positive bacteria *S. aureus* or to the Gram-negative bacteria *E. coli*.

Trained monocytes showed enhanced killing ability with respect to non-trained monocytes (Fig. 5C). Particularly, chitin-trained monocytes showed the most potent antibacterial killing. This result demonstrates an enhancement of direct antibacterial killing capacity of monocytes after induction of trained immunity by *S. cerevisiae*, and this effect is driven by chitin.

As the internalization ability of chitin-trained immune cells did not differ from one of not-trained cells (Figs. 6A-B), in order to investigate the events following internalization by monocytes, we evaluated the fate of the yeast-containing phagosomes in terms of acidification. As shown in Figs. 6C-D, the percent of *E. coli* as well as *C. albicans* containing-acid phagolysosomes was increased in chitin-trained cells with respect to not-trained monocytes, thus accounting for the more efficient ability to kill the pathogens.

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Discussion

Although an anti-inflammatory potential of microbial/yeast strains was occasionally suggested *in vitro* (25, 26) or in experimental models (25, 26), few reliable observations were reported in terms of strain variability and of specific mechanisms involved (27, 28). To our knowledge, no studies addressed *S. cerevisiae* immune reactivity at a strain level and its ability to modulate the cytokines response of human primary cells to bacteria and fungi. The different *S. cerevisiae* isolates tested induce trained immunity in monocytes, resulting in an enhanced pro-inflammatory cytokine production when they are exposed to a secondary bacterial or fungal stimulus.

The induction of trained immunity is dependent on the carbohydrate components of the cell wall of *S. cerevisiae*. This is in agreement with previous studies showing that β -glucan is an immunostimulant and is responsible for the training properties of *C. albicans* (14, 29). One of the main receptors mediating the priming effects of *S. cerevisiae* β -glucan is dectin-1, as shown by inhibitory experiments as well as the use of cells genetically deficient

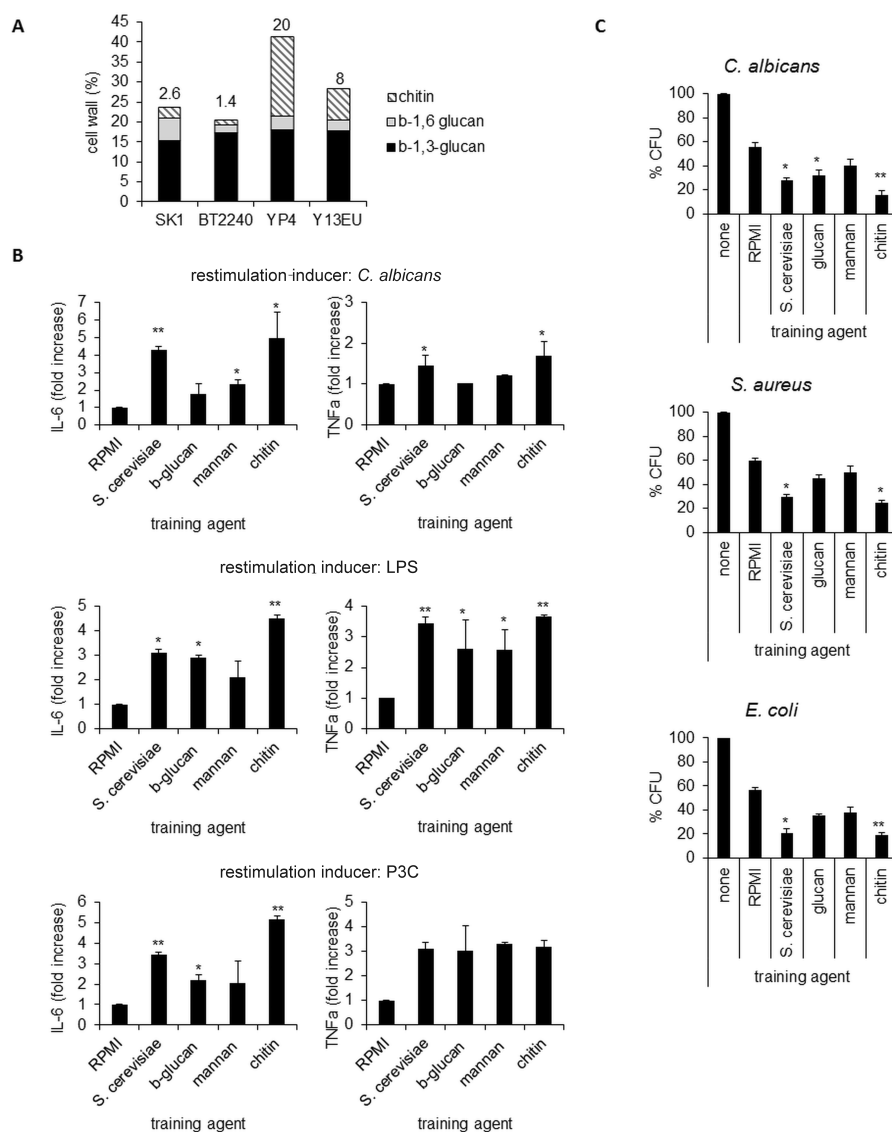


Fig. 5. Chitin drives training immune properties of *S. cerevisiae* and enhanced intracellular killing of primed monocytes. **A.** Cell wall composition characterization of the laboratory SK1 strain, the environmental BB1533 strain, the CD isolate YP4 and the healthy isolated Y13EU strain. Sugars composition is expressed in percentage of the total cell wall. Numbers on the top refer to the percentage of chitin content. **B.** Monocytes were exposed to RPMI, *S. cerevisiae* whole cell, *S. cerevisiae* derived chitin (10 μ g/ml), β -glucan (10 μ g/ml), mannan (10 μ g/ml) or a combination of mannan and β -glucan for 24 h, after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated with pure ligands or *C. albicans*. TNF- α and IL-6 were measured in supernatants. Data are presented as fold increase with respect to not-trained (RPMI-stimulated) cells. **C.** Survival of *C. albicans* and Gram⁺ (*S. aureus*) and Gram⁻ (*E. coli*) bacteria after killing by trained monocytes. Data are expressed as percentage of CFU of microorganisms growing after 6 h exposure. * $p < 0.05$, ** $p < 0.01$ trained cells versus RPMI-stimulated cells, Wilcoxon nonparametric test for two related samples. For (A), $n = 3$; for (B), (C), $n = 4$. Bars indicate mean \pm SD. LPS, lipopolysaccharyde; P3C, Pam₃Cys₄.

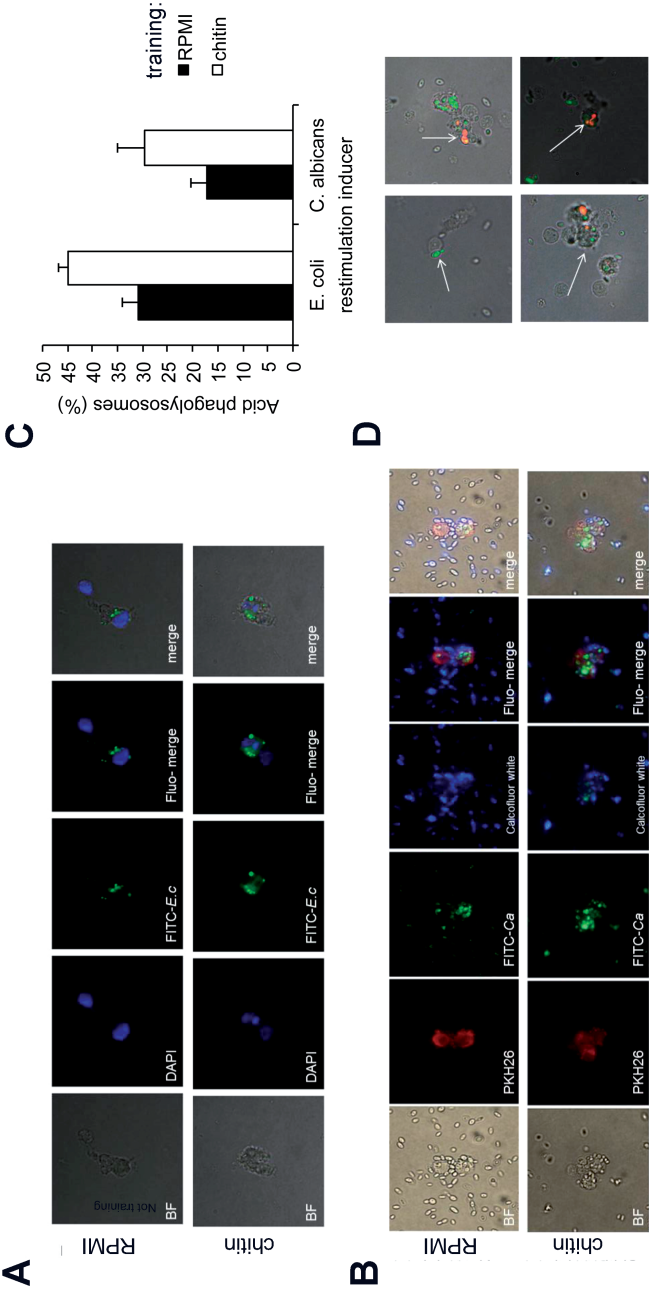


Fig. 6. Chitin does not change internalization properties but promotes killing by increasing the acidification of microbial containing phagolysosomes. Monocytes were exposed to RPML or *S. cerevisiae* derived chitin (10 μ g/ml) for 24 h, after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated for 1.5 h to green-labeled *E. coli* (A) or *C. albicans* (B). After appropriate staining, cells were visualized by fluorescence microscopy. Representative images of 3 independent experiments. C. Oregon green 488 prelabelled microorganisms were exposed to Lyso-Tracker labeled monocytes (E : T=1 : 5); samples were then fixed and visualized by fluorescence microscopy. The results, expressed as percent of acid phagolysosomes, were calculated by evaluating the number of red-stained vacuoles among 200 yeast-containing vacuoles. D. Representative images of *C. albicans* containing- acid phagolysosomes. Bars indicate mean \pm SD (N=3). * p <0.05 trained cells versus RPML-stimulated, Wilcoxon nonparametric test for two related samples.

for this receptor. However, the incomplete abolishment of training in monocytes genetically deficient for a functional dectin-1 receptor suggests that part of the trained immunity signal is mediated by other cell wall components.

Cell wall characterization in terms of β -glucan and chitin content indicated how isolates from different ecological niches showed a remarkable different cell wall composition, with the clinical isolates being highly enriched in chitin content. Under normal growth condition, chitin is a minor component of *C. albicans* cell wall, comprising only the 2-3% of its dry weight (30); however, chitin content of a *S. cerevisiae* isolate from a patient with Crohn's disease represented 20% of the dry weight of the yeast. Our results show that strains specifically isolated from CD patients (13) are able to strongly modulate cytokines response to bacterial antigens and fungi and this effect is largely dependent on chitin. Because training is induced towards purified TLR ligands, which are important components of bacteria, we tend to conclude that the priming induced by the diverse *S. cerevisiae* strains acts on TLR-dependent–signaling pathways, as shown by the reliable effect on Pam₃Cys₄ stimulation.

Even though chitin is an essential structural polysaccharide of fungal pathogens and parasites, some controversy persists on its role in human immune response (31–34). Earlier studies have shown that *C. albicans*-derived chitin reduces direct stimulation of *in vivo* LPS-induced inflammation and contributes to dampening the immune response by NOD2 and TLR9-mediated secretion of IL-10 (34). In a previous finding we showed how *S. cerevisiae* spores, which wall is largely composed by chitin, elicit inflammatory IL-17 responses, while cells of the same strain induce tolerance (21). Thus, by increasing the chitin content in their cell wall, fungi may influence the immune system in two ways: 1. favoring their persistence by influencing immune homeostasis and 2. training the innate host response against bacteria.

The results presented here give a novel perspective on the role of non-pathogenic microorganisms for immune homeostasis. The chitin-induced training suggests how chitin levels could importantly help breaking potential dysbiosis, priming organismal protection against bacterial pathogens. This training effect does not require colonization but can be elicited simply via exposure to chitin rich yeast cells or spores. Thus yeasts would play a fundamental role in shaping gut microbiota simply when passengers, rather than necessarily persisting as colonizers. We can speculate that the recent elimination of *S. cerevisiae*, as well as of other immune regulators, from fermented foods, and overall the reduction of early exposure to beneficial microbiomes (or mycobiomes?), is not only causing a reduction in anti-inflammatory signals, but is more importantly causing a decreased ability of “training” our immune system to handle pathogenic microorganisms, resulting in potentially uncontrolled immune reactions. The finding that the best “trainers” are strains isolated from Crohn's patients leads us to hypothesize an evolutionary advantage of chitin production in surviving and colonizing the human gut. It remains unclear if, in addition to these potential beneficial effects, induction of trained immunity may also have unintended deleterious effects, since fungi have been reported to increase the severity of intestinal inflammation in murine models of colitis (35,36).

Several directions of research are opened by this study: on the one hand future investigations should assess the molecular pathways that are involved in the induction

of trained immunity by *S. cerevisiae* and chitin, as recently shown to involve epigenetic (37) and metabolic (38) reprogramming for β -glucan, and on the other hand translational studies should assess the clinical potential of these effects. Further studies on the ability to induce trained immunity of yeasts in Crohn's mouse model will reveal whether trained immunity responses to fungi are potentially associated with onset of remission of Crohn's disease. Discovering the factors that drive microbial colonization will help us develop new antimicrobial therapeutics based on their potential to induce trained immunity. Selected strains of the 'Generally Recognized as Safe' *S. cerevisiae* described in this study could be specially suited for this task and for developing novel therapeutic approaches to IBD, or in general, to disease associated to inflammatory responses towards an unbalanced microbiota.

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L.R, D.C and M.G.N conceived the idea; L.R., D.C.I., J.Q., S.C. Cheng performed the experiments; C.D.F and M.S. provided the yeast strains; all the authors participated in results discussion; L.R. M.G.N and D.C wrote the manuscript.

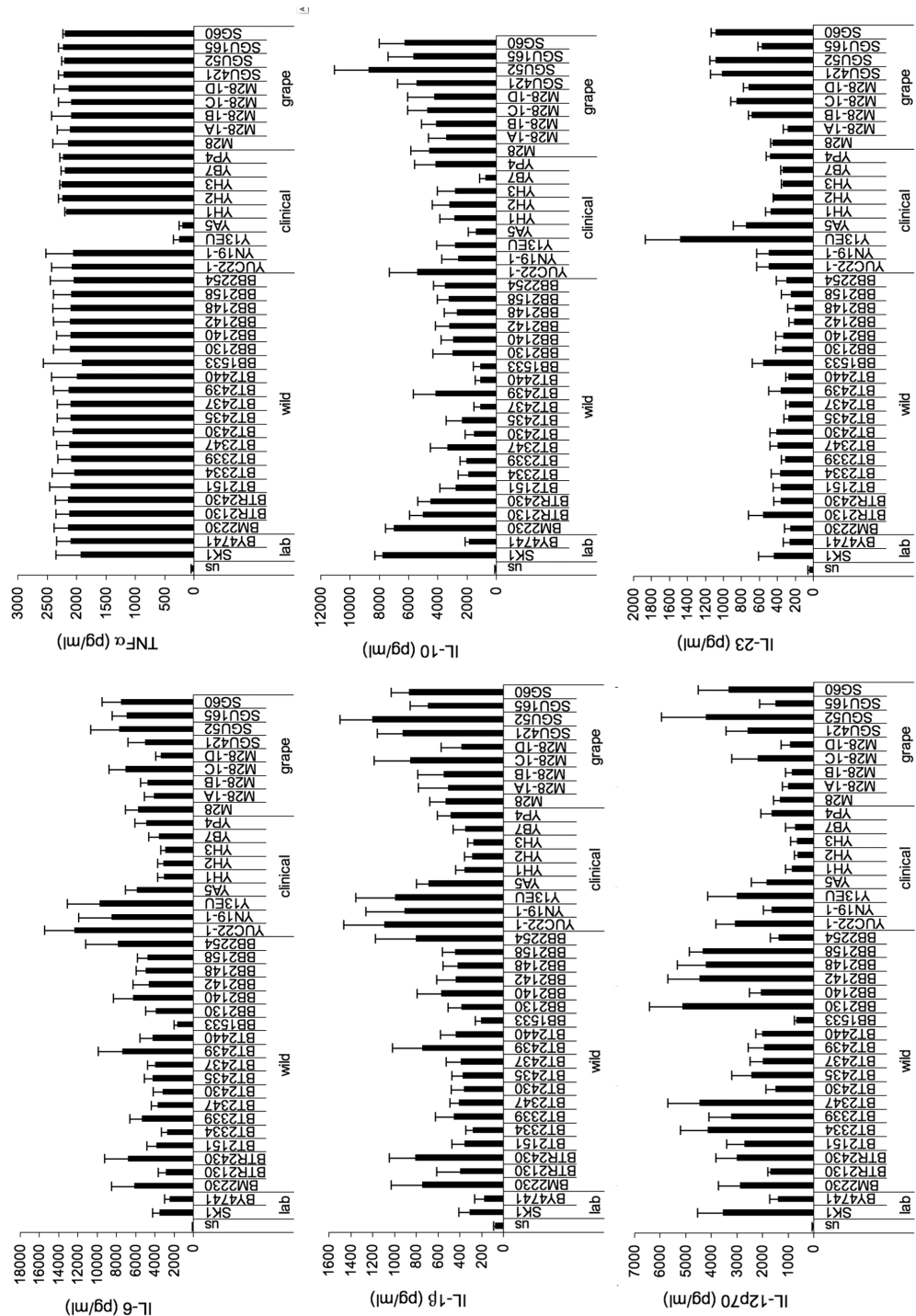
Conflict of Interest Disclosure:

The authors declare no commercial or financial conflict of interest.

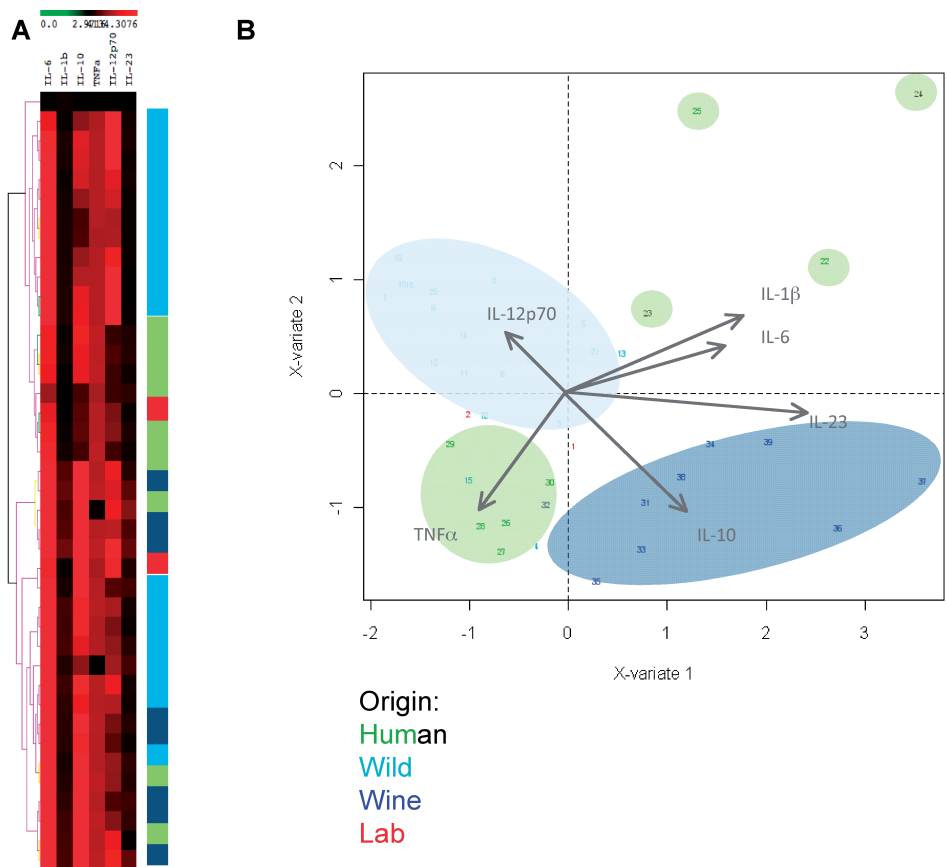
References

- Durrant WE, Dong X. Systemic acquired resistance. *Annu Rev Phytopathol*. 2004;42:185–209.
- Conrath U. Molecular aspects of defence priming. *Trends Plant Sci*. 2011 Oct;16(10):524–31.
- Kurtz J. Specific memory within innate immune systems. *Trends Immunol*. 2005 Jan 4;26(4):186–92.
- Quintin J, Cheng S-C, van der Meer JW, Netea MG. Innate immune memory: towards a better understanding of host defense mechanisms. *Curr Opin Immunol*. 2014 Aug;29:1–7.
- Netea MG, Quintin J, van der Meer JWM. Trained Immunity: A Memory for Innate Host Defense. *Cell Host Microbe*. 2011 May 19;9(5):355–61.
- Bonifazi P, Zelante T, D'Angelo C, De Luca A, Moretti S, Bozza S, et al. Balancing inflammation and tolerance in vivo through dendritic cells by the commensal *Candida albicans*. *Mucosal Immunol*. 2009 Jul;2(4):362–74.
- Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, et al. Differential adaptation of *Candida albicans* in vivo modulates immune recognition by dectin-1. *PLoS Pathog*. 2013;9(4):e1003315.
- Rizzetto L, Giovannini G, Bromley M, Bowyer P, Romani L, Cavalieri D. Strain dependent variation of immune responses to *A. fumigatus*: definition of pathogenic species. *PLoS One*. 2013;8(2):e56651.
- Oh J, Freeman AF, NISC Comparative Sequencing Program, Park M, Sokolic R, Candotti F, et al. The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. *Genome Res*. 2013 Dec;23(12):2103–14.
- Romani L, Zelante T, Palmieri M, Napolioni V, Picciolini M, Velardi A, et al. The cross-talk between opportunistic fungi and the mammalian host via microbiota's metabolism. *Semin Immunopathol*. 2015 Mar;37(2):163–71.
- Romani L, Zelante T, De Luca A, Iannitti RG, Moretti S, Bartoli A, et al. Microbiota control of a tryptophan-AhR pathway in disease tolerance to fungi. *Eur J Immunol*. 2014 Nov;44(11):3192–200.
- Mukherjee PK, Sendid B, Hoarau G, Colombel J-F, Poulain D, Ghannoum MA. Mycobiota in gastrointestinal diseases. *Nat Rev Gastroenterol Hepatol*. 2015 Feb;12(2):77–87.
- De Filippo C, Di Paola M, Stefanini I, Rizzetto L, Berná L, Ramazzotti M, et al. Population genomics of *Saccharomyces cerevisiae* human isolates: passengers, colonizers, invaders. *bioRxiv* [Internet]. 2014 Jan 17; Available from: <http://biorxiv.org/content/early/2014/01/17/001891.abstract>
- Ifrim DC, Joosten LAB, Kullberg B-J, Jacobs L, Jansen T, Williams DL, et al. *Candida albicans* primes TLR cytokine responses through a Dectin-1/Raf-1-mediated pathway. *J Immunol Baltim Md 1950*. 2013 Apr 15;190(8):4129–35.
- Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe*. 2012 Aug 16;12(2):223–32.
- Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA, et al. Human Skin Fungal Diversity. *Nature*. 2013 Jun 20;498(7454):367–70.
- Xu J, Boyd CM, Livingston E, Meyer W, Madden JF, Mitchell TG. Species and genotypic diversities and similarities of pathogenic yeasts colonizing women. *J Clin Microbiol*. 1999 Dec;37(12):3835–43.
- Angebault C, Djossou F, Abélanet S, Permal E, Ben Soltana M, Diancourt L, et al. *Candida albicans* is not always the preferential yeast colonizing humans: a study in Wayampi Amerindians. *J Infect Dis*. 2013 Nov 15;208(10):1705–16.
- Kane SM, Roth R. Carbohydrate metabolism during ascospore development in yeast. *J Bacteriol*. 1974 Apr;118(1):8–14.
- Baker Brachmann C, Davies A, Cost GJ, Caputo E, Li J, Hieter P, et al. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. 1998 Jan 30;14(2):115–32.
- Rizzetto L, Kuka M, Filippo CD, Cambi A, Netea MG, Beltrame L, et al. Differential IL-17 Production and Mannan Recognition Contribute to Fungal Pathogenicity and Commensalism. *J Immunol*. 2010 Apr 15;184(8):4258–68.
- Landry CR, Townsend JP, Hartl DL, Cavalieri D. Ecological and evolutionary genomics of *Saccharomyces cerevisiae*. *Mol Ecol*. 2006 Mar;15(3):575–91.
- Mahla RS, Reddy MC, Prasad DVR, Kumar H. Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine

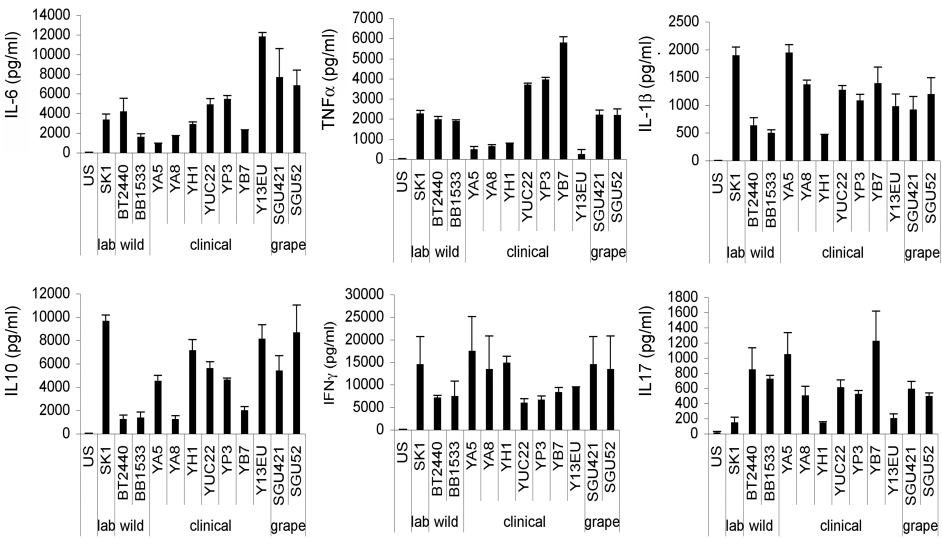
- Biology. Front Immunol [Internet]. 2013 Sep 2 [cited 2015 Jan 5];4. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3759294/>
- 24 Van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJPM, Cheng S-C, Joosten I, et al. The Macrophage Mannose Receptor Induces IL-17 in Response to *Candida albicans*. *Cell Host Microbe*. 2009 Apr 23;5(4):329–40.
 - 25 Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Järvenpää S, et al. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World J Gastroenterol WJG*. 2008 Apr 7;14(13):2029–36.
 - 26 Foligne B, Nutton S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol WJG*. 2007 Jan 14;13(2):236–43.
 - 27 Foligné B, Deutsch S-M, Breton J, Cousin FJ, Dewulf J, Samson M, et al. Promising Immunomodulatory Effects of Selected Strains of Dairy Propionibacteria as Evidenced In Vitro and In Vivo. *Appl Environ Microbiol*. 2010 Dec;76(24):8259–64.
 - 28 Foligné B, Dewulf J, Vandekerckove P, Pignède G, Pot B. Probiotic yeasts: Anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice. *World J Gastroenterol WJG*. 2010 May 7;16(17):2134–45.
 - 29 Vetvicka V. Glucan-immunostimulant, adjuvant, potential drug. *World J Clin Oncol*. 2011 Feb 10;2(2):115–9.
 - 30 Klis FM, de Groot P, Hellingwerf K. Molecular organization of the cell wall of *Candida albicans*. *Med Mycol*. 2001;39 Suppl 1:1–8.
 - 31 Alvarez FJ. The effect of chitin size, shape, source and purification method on immune recognition. *Mol Basel Switz*. 2014;19(4):4433–51.
 - 32 Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J Immunol Baltim Md 1950*. 2009 Mar 15;182(6):3573–82.
 - 33 Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, et al. Recognition and blocking of innate immunity cells by *Candida albicans* chitin. *Infect Immun*. 2011 May;79(5):1961–70.
 - 34 Wagener J, Malireddi RKS, Lenardon MD, Köberle M, Vautier S, MacCallum DM, et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog*. 2014 Apr;10(4):e1004050.
 - 35 Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. 2012 Jun 8;336(6086):1314–7.
 - 36 Poulain D, Sendid B, Standaert-Vitse A, Fradin C, Jouault T, Jawhara S, et al. Yeasts: neglected pathogens. *Dig Dis Basel Switz*. 2009;27 Suppl 1:104–10.
 - 37 Saeed S, Quintin J, Kerstens HHD, Rao NA, Aghajaniirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014 Sep 26;345(6204):1251086.
 - 38 Cheng S-C, Quintin J, Cramer RA, Shephardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity. *Science*. 2014 Sep 26;345(6204):1250684.
 - 39 Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spruel AB, Venselaar H, et al. Human Dectin-1 Deficiency and Mucocutaneous Fungal Infections. *N Engl J Med*. 2009 Oct 29;361(18):1760–7.
 - 40 Lowman DW, Ensley HE, Greene RR, Knagge KJ, Williams DL, Kruppa MD. Mannan structural complexity is decreased when *Candida albicans* is cultivated in blood or serum at physiological temperature. *Carbohydr Res*. 2011 Dec 13;346(17):2752–9.
 - 41 Müller A, Rice PJ, Ensley HE, Coogan PS, Kalbfleisch JH, Kelley JL, et al. Receptor binding and internalization of a water-soluble (1 \rightarrow 3)- β -D-glucan biologic response modifier in two monocyte/macrophage cell lines. *J Immunol Baltim Md 1950*. 1996 May 1;156(9):3418–25.
 - 42 Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol Baltim Md 1950*. 2000 Jul 15;165(2):618–22.
 - 43 Ferreira C, Silva S, van Voorst F, Aguiar C, Kielland-Brandt MC, Brandt A, et al. Absence of Gup1p in *Saccharomyces cerevisiae* results in defective cell wall composition, assembly, stability and morphology. *FEMS Yeast Res*. 2006 Nov;6(7):1027–38.



◀ **Supplementary Fig. 1:** Immune reactivity to different *S. cerevisiae* isolates. The ability of DCs to discriminate among different strains of the *S. cerevisiae* species was tested as differential cytokine production. DCs were stimulated with yeast cells for 24 h and cultures supernatants used for TNF- α , IL-10, IL-1 β , IL-6, IL-12p70 and IL-23 measurements. TNF α was fairly uniform for the different strains investigated, with the exception of two clinical isolates – one derived from a healthy subject (Y13EU), one from a patient (YA5) - which showed a markedly low induction of this cytokine. Clinical isolates from patients showed a lower induction of IL-12p70 with respect to the healthy human isolates. Despite differences in IL-12p70, the Tuscan grape isolates induced uniform levels of IL-10. Immune responses induced by wild Portuguese strains were more variable. Interestingly, the wild BB1533 strain induced a response similar to some clinical isolates. This strain also showed clear growth advantages on Cycloheximide, Paramomycin, Benomyl, 4-nitroquinoline oxide and vanadate in a phenomic screen and was genetically distinct from the other *S. cerevisiae* isolates (Santos M., unpublished observations). These results highlight the ability of *S. cerevisiae* to promote both, pro- or anti-inflammatory responses upon immune cell recognition. Bars indicate mean \pm SD (n=6).



Supplementary Fig. 2: Immune reactivity to different *S. cerevisiae* isolates correlates with their origin. (A) Clustering of cytokine production induced by moDCs in response to different *S. cerevisiae* strains. Pearson correlation calculated using TMeV software. B. Principal components analysis (PCA) over cytokine response to different *S. cerevisiae* strains.



Supplementary Fig. 3: Immune reactivity to different *S. cerevisiae* isolates. Healthy PBMCs were stimulated with various *S. cerevisiae* strains for 24 h or 5 days, and cytokine protein levels were measured in the supernatants. Bars indicate mean + SD (n = 8).



**Defective trained immunity
in patients with STAT1-dependent
chronic mucocutaneous candidiasis**

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Abstract

Patients with *STAT1*-dependent chronic mucocutaneous candidiasis (CMC) and patients with *STAT3*-dependent hyper IgE syndrome (HIES) display defects in Th17 cytokine production capacity. Despite this similar immune defect in Th17 function, they show important differences in the type of infections to which they are susceptible. Recently, our group reported differential regulation of STAT1 and STAT3 transcription factors during epigenetic reprogramming of trained immunity, an important host defense mechanism based on innate immune memory. We therefore hypothesized that *STAT1* and *STAT3* defects have different effects on trained immunity and this may partly explain the differences between CMC and HIES regarding the susceptibility to infections. Indeed, while trained immunity was normally induced in cells isolated from patients with HIES, the induction of innate training was defective in CMC patients. This defect was specific for training with *Candida albicans*, the main pathogen encountered in CMC, and it involved a type II interferon-dependent mechanism. These findings describe the role of STAT1 for the induction of trained immunity, and may contribute to the understanding of the differences in susceptibility to infection between CMC and HIES patients. This study could also provide directions for personalized immunotherapy in patients suffering from these immunodeficiencies.

Keywords: trained immunity, CMC, HIES

Introduction

Chronic Mucocutaneous Candidiasis (CMC) and hyper immunoglobulinemia E syndrome (HIES) are rare primary immunodeficiencies, both characterized by defective Th17 cytokine production; this immunological defect is mainly caused by *STAT1* and *STAT3* mutations, respectively (1-5). More rarely, mutations in *IL-17F* or *IL-17RA* have also been described in CMC patients (6), and *DOCK8* mutations in autosomal recessive HIES (7). Despite the similarities between CMC and HIES concerning genetic and immunological defects, important phenotypic differences distinguish these clinical entities regarding susceptibility to infections. While CMC patients suffer mainly from *Candida* and dermatophyte infections, and only later during life from respiratory tract infections (8), HIES patients have an increased susceptibility to skin and respiratory tract infections, especially with *Staphylococcus aureus* and *Streptococcus pneumoniae*, while their fungal complications are less prominent (4, 9-12). The immunological substrate for these differences in susceptibility is not known. Other striking differences are the skeletal abnormalities in HIES and the autoimmune endocrinopathy in CMC (13, 14).

Recently, a novel host defense mechanism has been proposed, consisting of long-term enhanced biological activity of innate immune cells, which was termed *trained immunity* (15, 16). Trained immunity relies on epigenetic reprogramming of monocytes or macrophages, and represents a *de-facto* memory of the innate immune response (17, 18). The epigenetic and metabolic substrate of trained immunity has been lately described (19, 20). Interestingly, these studies uncovered differential regulation of *STAT1* and *STAT3* during induction of trained immunity and innate immune tolerance (19). This finding led us to hypothesize that the induction of trained immunity may be differentially regulated or defective in patients suffering from CMC and/or HIES.

In the present study we explored the trained immunity responses in peripheral blood mononuclear cells isolated from either CMC or HIES patients. We demonstrate that CMC patients display a defective induction of trained immunity by *Candida albicans*, while this was normal in HIES patients. This defect in CMC patients is specific for *Candida* and is mediated through an interferon- γ (IFN- γ)-dependent pathway. These findings might explain the incapacity of CMC patients to clear fungal infections, and may lead to further understanding the differences between these two complex immunodeficiency disorders.

Materials and Methods

Patients and healthy volunteers. Blood samples were collected from five autosomal dominant CMC patients harboring mutations in the *STAT1* gene, three HIES patients with *STAT3* mutations, and from age- and gender-matched healthy volunteers who did not suffer from infectious or inflammatory diseases. Blood was collected by venipuncture into 10 mL EDTA tubes (367525; BD). The study was approved by the Arnhem-Nijmegen Ethical Committee, and the participants gave written informed consent.

Reagents and microorganisms. Culture medium used was RPMI 1640 Dutch modifications (Sigma-Aldrich) supplemented with 1% gentamicin, 1% L-glutamine and 1% pyruvate (Life Technologies, Nieuwekerk, Netherlands). Particulate *C. albicans* β -1,3-glucan was isolated and purified as previously described (21). *C. albicans* ATCC MYA-3573 (UC 820) (22) was grown overnight in Sabouraud broth at 29°C; cells were harvested by centrifugation, washed twice, and resuspended in PBS. *Candida* conidia were heat killed for 30 min at 95 °C. Other reagents were obtained as follows: LPS (*E. coli* serotype 055:B5, Sigma-Aldrich) with an additional purification step (23), CD56 MicroBeads were purchased from Miltenyi Biotec. Ustekinumab (CNTO 1275) was purchased from Janssen Biotech, Inc. Nanogam (IgG, human normal immunoglobulin, IVIg) was purchased from Sanquin and IL-18BP was kindly provided by Prof. Charles Dinarello (University of Colorado).

Stimulation experiments. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (24). PBMCs were counted on a Beckman Coulter Z1 Particle Counter and adjusted to 5×10^6 cells/ml. The trained immunity protocol was performed as described (17): in brief, 100 μ l of the PBMC suspension were added per well in 96-well round-bottom plates, and pre-incubated for 24 h with either RPMI, β -glucan (1 μ g/ml) or heat-killed *Candida* conidia 10^4 CFU/ml, at 37 °C and 5% CO₂. After preincubation, cells were washed with warm PBS and let to rest in RPMI supplemented with 10% human pool serum for 5 days (period during which media was refreshed on day 3). Thereafter, on day 6, cells were subjected to a second stimulation of cytokine production with various stimuli (RPMI, LPS 10 ng/ml, or *Candida* 10^5 CFU/ml) in a final volume of 200 μ l. After another 24 hours, supernatants were collected and stored at -20 °C until assayed. To investigate whether IFN- γ inhibitors affect *Candida* training, PBMCs were preincubated for 1 h prior to the first stimulation with IgG control, Ustekinumab or IL-18BP in a final concentration of 10 μ g/ml. Subsequently, RPMI or *Candida* were added to the cells with the inhibitors for an additional 24 h. Thereafter, PBMCs were washed with PBS and further incubated for 5 days in culture medium supplemented with 10% pooled human serum. On day 6, the trained macrophages were subjected to a second stimulation, for 24 h, with either LPS or *Candida*. The supernatants were collected and stored at -20°C until assessed.

To assess the role of NK cells in this process, NK cells were isolated via positive selection with CD56 isolation beads from Miltenyi Biotec conform manufacturer's instructions. Thereafter, the CD56 positive cells were stimulated for 48 hours with heat-killed *Candida* (10^6 CFU/ml) or heat-killed *S. aureus* (10^7 CFU/ml). PBMCs depleted of NK cells were further included in training experiments, being trained with *Candida* (10^4 CFU/ml) as described above and restimulated with either LPS (10 ng/ml) or *Candida* (10^5 CFU/ml). After 24 hours of cell stimulation, supernatants were collected and stored at -20 °C until the amounts of TNF- α and IL-6 were assayed.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of TNF- α (R&D Systems, Abingdon, UK), and IL-6 (Sanquin, Amsterdam, Netherlands) were measured 24 h after the second stimulation in cell culture supernatants with ELISA, according to

the manufacturer instructions. The concentration of IFN- γ (Sanquin, Amsterdam, Netherlands) was measured in cell culture supernatants at 48h after the stimulation.

Statistical analysis. Results from at least two sets of experiments with a minimum of four volunteers were pooled and analyzed in GraphPad. The data are presented as the means \pm SEM and Wilcoxon signed-rank test was used to compare differences between groups. The level of significance was set at $*p < 0.05$.

Results

PBMCs isolated from patients with CMC, but not HIES, display defective *Candida*-mediated trained immunity

As previously shown, PBMCs of patients who suffer from CMC due to *STAT1* mutations stimulated for 24 h with heat-killed *Candida* were able to produce normal amounts of TNF- α and IL-6 compared to the healthy controls (2). We therefore tested whether trained immunity could be triggered by *Candida*. PBMCs from five CMC patients and nine healthy donors that were exposed to β -glucan, *Candida* and RPMI (negative control) for 24 h. On day 6 after recovery, cells were subjected to a second exposure to LPS, *Candida* or RPMI (negative control) for an additional 24 h. Interestingly, while PBMCs from CMC patients could be trained by β -glucan (Suppl. Fig. 1), they could not be trained by *C. albicans* (Fig. 1).

In contrast, PBMCs isolated from HIES patients showed a normal degree of training with both *Candida* (Fig. 2) and β -glucan (Suppl. Fig. 2), as shown by the enhanced production of TNF- α and IL-6 upon restimulation with either LPS or *Candida*.

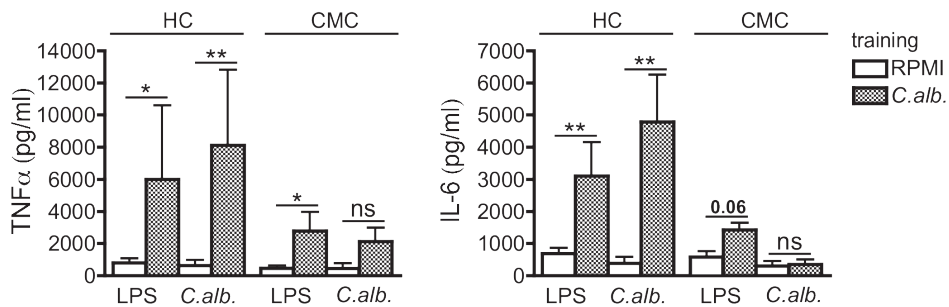


Figure 1: Defective trained immunity in CMC patients. PBMCs from healthy volunteers and CMC patients were trained for 24 hours by incubation with culture medium or low concentrations of *C. albicans* (10^4 CFU/ml). Thereafter the stimulus was removed and cells were let to rest in RPMI with 10% human serum. On day 6, a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls, $n = 5$ CMC patients. The data are presented as the means \pm SEM. HC: healthy control; CMC: Chronic Mucocutaneous Candidiasis; LPS: Lipopolysaccharide; *C. alb.*: *Candida albicans*.

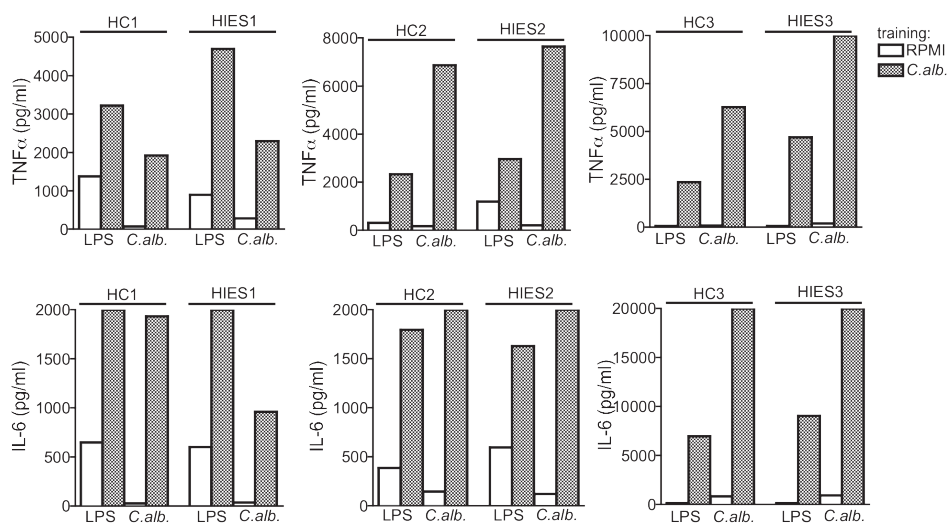


Figure 2: Normal trained immunity in HIES patients. PBMCs from healthy volunteers and HIES patients were trained for 24 hours with culture medium or to very low concentrations of *C. albicans* (10^4 CFU/ml). At day 6, a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls and $n = 3$ HIES patients. The data are presented as the means \pm SEM. HC: healthy control; HIES: Hyper-IgE syndrome; LPS: Lipopolysaccharide; *C. alb.*: *Candida albicans*.

Defective IFN- γ production by NK cells from CMC patients

The defective trained immunity in PBMCs from CMC patients suggests that STAT1, but not STAT3, is crucial for *Candida*-induced trained immunity. IFN- γ is a proinflammatory cytokine produced by NK- and T-cells that induces activation of both innate and adaptive immunity against candidiasis. Because IFN- γ acts through an IFNGR- and STAT1-dependent signaling pathway, and has been previously reported to epigenetically reverse immune tolerance (25), we hypothesized that it may be involved in the defective trained immunity in CMC. Interestingly, while purified NK cells from HIES patients produced similar amounts of IFN- γ as the healthy controls (Fig. 3A), IFN- γ production was significantly diminished in CMC patients (Fig. 3A), suggesting defective NK cell function in patients suffering of CMC only. Moreover, when PBMCs from both groups of patients were depleted of NKs and subsequently trained with *Candida*, not only the cells from patients with CMC showed a defect in training, but NK-depleted cells from HIES were also not able to respond to a similar extend as PBMCs from healthy controls (Fig. 3B), demonstrating an essential role for the NK-cell-derived IFN- γ production for the induction of training in HIES patients. Of importance, depletion of NK cells in PBMCs from healthy volunteers did not abolish their capacity to mount a trained immunity response (Fig. 3C), suggesting that in healthy volunteers T-cell-derived IFN- γ is able to complement for the loss of NK-cell function. The defective Th1-cell function in CMC and HIES patients leads however to a strong dependency on NK-cell-derived IFN- γ .

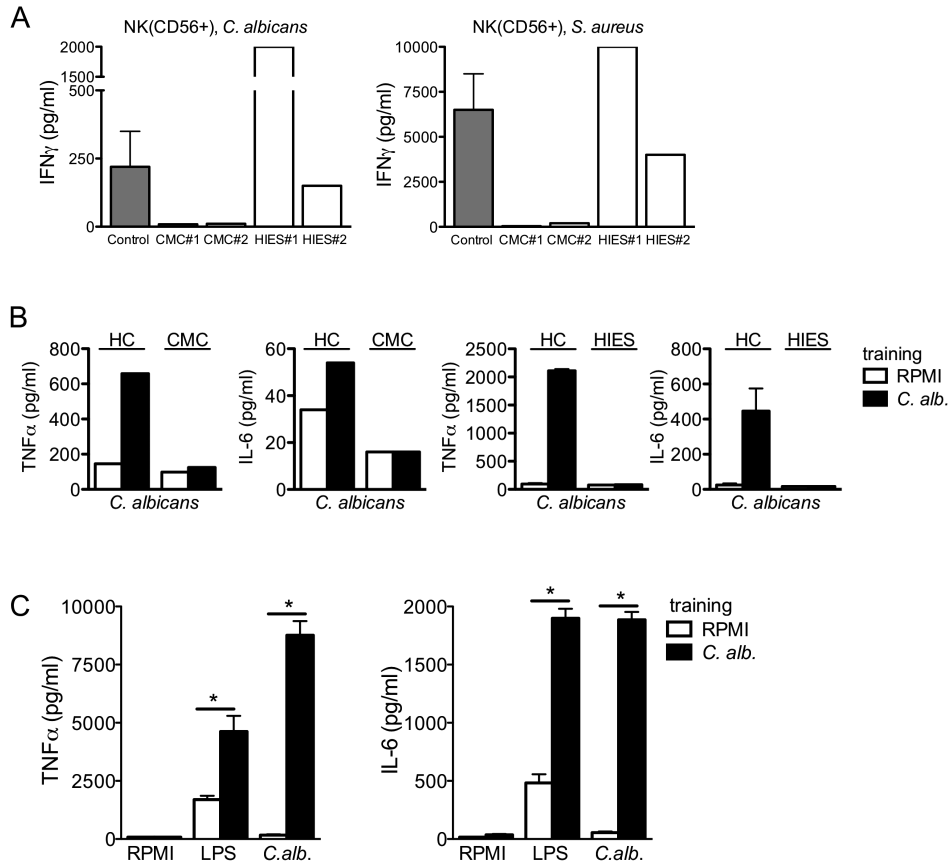


Figure 3: NK-dependent production of IFN- γ mediated trained immunity. (A) Defective NK cells in CMC patients. NK cells from two CMC and two HIES patients were isolated with CD56 positive beads and thereafter stimulated with either *C. albicans* or *S. aureus*. IFN- γ was measured in cell culture supernatants after 48 hours of incubation. (B) Defective training of PBMCs depleted of NK cells in CMC and HIES patients. PBMCs from one CMC and two HIES patients were first depleted of NK cells and then trained for 24 hours with culture medium or to very low concentrations of *C. albicans* (10^4 CFU/ml). At day 6, a second stimulation with *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. The data are presented as the means \pm SEM. HC: healthy control; CMC: Chronic Mucocutaneous Candidiasis; HIES: Hyper-IgE syndrome; *C.alb.*: *Candida albicans*. (C) Training of PBMCs from healthy controls depleted of NK cells. PBMCs from healthy controls were trained for 24 hours with culture medium or with very low concentrations of *C. albicans* (10^4 CFU/ml), after which they were stimulated as described above. $n = 6$, 3 independent experiments. The data are presented as the means \pm SEM. LPS: Lipopolysaccharide; *C.alb.*: *Candida albicans*.

Inhibiting IFN- γ production by blocking IL-12 and IL-23 with Ustekinumab, or by blocking IL-18 with IL-18BP, significantly decreased the training by *Candida* in PBMCs from healthy controls, both for TNF- α and IL-6 (Fig. 4). Of note, the training was not abolished by IgG control (Fig. 4). Moreover, the inhibition was significant only for *Candidia* training and *Candidia* restimulation, and not for LPS restimulation (data not shown).

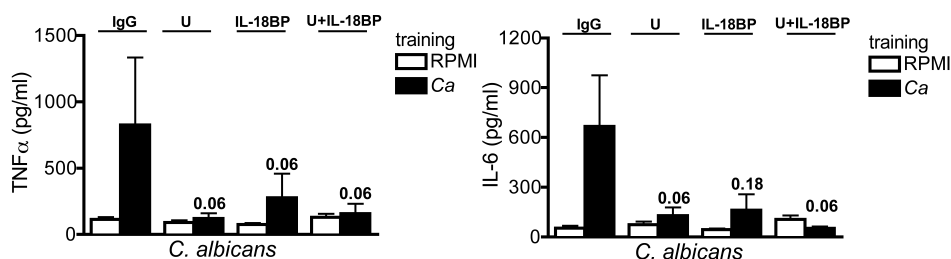


Figure 4: IFN- γ -dependent *Candida*-induced training. Before training, PBMCs were preincubated for 1 h with IgG (isotype control) (10 μ g/ml), Ustekinumab (10 μ g/ml) or IL-18BP (10 μ g/ml). Thereafter, 100 μ l of culture medium or *C. albicans* (10^4 CFU/ml) was added to the cell suspension, without removing the inhibitors, and further incubated for an additional 23 h. At day 6, macrophages were subjected to a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) for another 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. Data are presented as mean \pm SEM ($n = 5$, three independent experiments), * $p < 0.05$. Wilcoxon signed rank test was used to detect significant differences. U: Ustekinumab; Ca.: *Candida albicans*.

Discussion

In this study we demonstrated that PBMCs from CMC patients failed to mount *Candida*-induced trained immunity. This process was caused by *STAT1* defects and mediated through NK-cell-dependent production of IFN- γ .

The findings of the present study are relevant at several levels. Firstly, they point to an important immunological difference between patients with CMC and HIES. As both these immunodeficiencies are caused by genetic defects in STAT molecules, and have a similar immunological defect in proper activation of Th17 pathway (1, 3), it remained unexplained why their susceptibility to infection is different. The identification of specific defects in *Candida*-induced trained immunity in CMC, but not HIES patients, represents a first clue for the severe susceptibility to fungal infections in CMC, compared with the less prominent susceptibility to fungal infections in HIES. Secondly, the discovery of trained immunity defects in CMC patients, and not in HIES, provides important insights into understanding the communication between important players in host defense. Through *in vitro* experiments with NK cells and NK cell depletions, we concluded that T- and NK cell-derived IFN- γ plays a central role in potentiating trained immunity in a paracrine and possible endocrine manner. Although trained immunity has been initially described to be induced in monocytes, it should be expected that important interactions should take place with other cell types. The first of such interactions is described here, with NK-cell derived IFN- γ playing a central role in potentiating trained immunity in a paracrine and possible endocrine manner. We found that while the role of NK-cells is redundant in healthy individuals with normal T-cell function, the release of IFN- γ by NK cells rescues the training in HIES patients that have defective T cell IFN- γ production. In contrast, CMC patients that display both defective T- and NK-cell production of IFN- γ explain defective trained immunity. A role for NK-cells in the induction of trained immunity has been also recently described after BCG vaccination (26).

The role of IFN- γ and STAT1 for the induction of trained immunity is in line with previous observations. We have described differential regulation of STAT1 and STAT3 during induction of trained immunity and immune tolerance (19), data that provided the initial impulse to study these mechanisms in CMC and HIES. Furthermore, IFN- γ has been demonstrated to reverse the effects of LPS tolerance, the ‘opposite’ of trained immunity, specifically by activating *Brg1*, which in turn remodels chromatin facilitating the access of transcription factors (25). IFN- γ exerts its biological activity through activation of intracellular pathways containing Jak1, Jak2 and STAT1 (27). The role of STAT1 signaling for trained immunity may thus be explained through its role in the type II IFNGR pathway. In line with this, a recent study demonstrated that activation of STAT-1 by IFN- γ correlates with multiple histone modifications such as H3K4me2, H3K4me3, H3K9me3 and H3K36me3 (28).

It remains unclear why β -glucan is still able to train cells from CMC patients, as it has been proposed that the mechanism through which *Candida* trains immune cells is through β -glucan/Dectin-1 (17). However, recent data suggesting that other cell wall *Candida* structures, such as chitin, are also important for trained immunity induced by the whole fungus (Rizzetto L, unpublished data) may represent one possible explanation. One limitation of the study that should be acknowledged is the limited number of patients. Unfortunately, this was unavoidable due to the rare nature of both CMC and HIES diseases.

Although the *in vitro* model of training PBMCs is a simplified system, it does provide a direct assessment of the propensity of PBMCs from diverse individuals to be trained. However, this is the first study reporting defective trained immunity in CMC and normal training effects in HIES patients. Thereby this study enhances our understanding of specific immunological disturbances in each of these complex immunodeficiencies. Moreover, it can provide new openings in the design of novel immunotherapeutical strategies towards a personalized treatment of CMC and HIES.

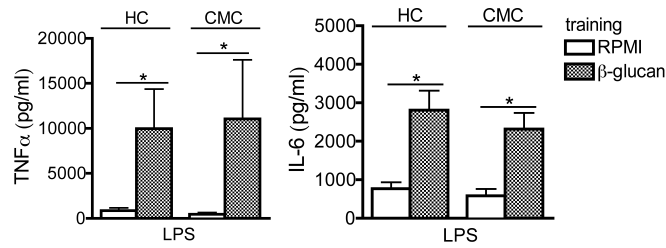
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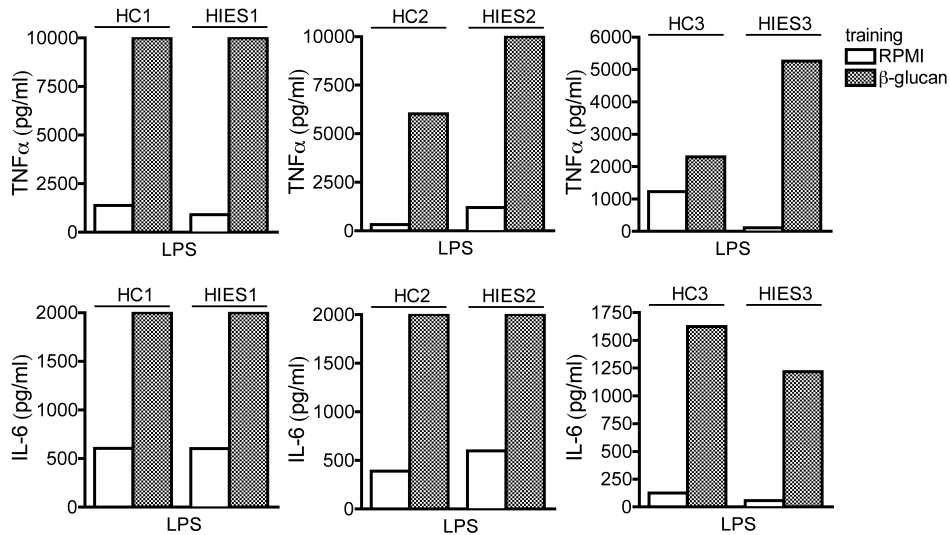
References

- 1 Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med*. 2009 Feb 16;206(2):299-311.
- 2 van de Veerdonk FL, Plantinga TS, Hoischen A, Smekens SP, Joosten LA, Gilissen C, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. *The New England journal of medicine*. [Research Support, Non-U.S. Gov't]. 2011 Jul 7;365(1):54-61.
- 3 Milner JD, Brechley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. 2008 Apr 10;452(7188):773-U11.
- 4 Ma CS, Chew GYJ, Simpson N, Priyadarshi A, Wong M, Grimbacher B, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. 2008 Jul 7;205(7):1551-7.
- 5 de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med*. 2008 Jul 7;205(7):1543-50.
- 6 Liu L, Okada S, Kong XF, Kreins AY, Cypowij S, Abhyankar A, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J Exp Med*. 2011 Aug 1;208(8):1635-48.
- 7 Xue L, Yang Y, Wang S. A novel large deletion of the DOCK8 gene in a Chinese family with autosomal-recessive hyper-IgE syndrome. *J Eur Acad Dermatol Venereol*. 2015 Feb 17.
- 8 Soltesz B, Toth B, Shabashova N, Bondarenko A, Okada S, Cypowij S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. *J Med Genet*. 2013 Sep;50(9):567-78.
- 9 Paulson ML, Freeman AF, Holland SM. Hyper IgE syndrome: an update on clinical aspects and the role of signal transducer and activator of transcription 3. *Curr Opin Allergy Clin*. 2008 Dec;8(6):527-33.
- 10 Freeman AF, Holland SM. Clinical Manifestations, Etiology, and Pathogenesis of the Hyper-IgE Syndromes. *Pediatr Res*. 2009 May;65(5):32r-7r.
- 11 Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, et al. STAT3 mutations in the hyper-IgE syndrome. *The New England journal of medicine*. [Research Support, N.I.H., Extramural Research Support, N.I.H., Intramural Research Support, Non-U.S. Gov't]. 2007 Oct 18;357(16):1608-19.
- 12 Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature*. 2007 Aug 30;448(7157):1058-U10.
- 13 Davis SD, Schaller J, Wedgwood RJ. Job's Syndrome. Recurrent, "cold", staphylococcal abscesses. *Lancet*. 1966 May 7;1(7445):1013-5.
- 14 Sowerwine KJ, Holland SM, Freeman AF. Hyper-IgE syndrome update. *Ann N Y Acad Sci*. 2012 Feb;1250:25-32.
- 15 Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell host & microbe*. [Research Support, Non-U.S. Gov't Review]. 2011 May 19;9(5):355-61.
- 16 Bowdish DM, Loffredo MS, Mukhopadhyay S, Mantovani A, Gordon S. Macrophage receptors implicated in the "adaptive" form of innate immunity. *Microbes Infect*. 2007 Nov-Dec;9(14-15):1680-7.
- 17 Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Iffrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell host & microbe*. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. 2012 Aug 16;12(2):223-32.
- 18 Quintin J, Cheng SC, van der Meer JW, Netea MG. Innate immune memory: towards a better understanding of host defense mechanisms. *Curr Opin Immunol*. 2014 Aug;29:1-7.
- 19 Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajani-Refah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014 Sep 26;345(6204):1251086.
- 20 Cheng SC, Quintin J, Cramer RA, Shephardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science*. 2014 Sep 26;345(6204):1250684.
- 21 Muller A, Rice PJ, Ensley HE, Coogan PS, Kalbfleisch JH, Kelley JL, et al. Receptor binding and internalization of a water-soluble (1->3)-beta-D-glucan biologic response modifier in two monocyte/macrophage cell

- lines. *Journal of immunology*. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]. 1996 May 1;156(9):3418-25.
- 22 Lehrer RI, Cline MJ. Interaction of *Candida albicans* with human leukocytes and serum. *Journal of bacteriology*. 1969 Jun;98(3):996-1004.
- 23 Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: Repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *Journal of immunology*. 2000 Jul 15;165(2):618-22.
- 24 Endres S, Ghorbani R, Lonnemann G, van der Meer JW, Dinarello CA. Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin Immunol Immunopathol*. 1988 Dec;49(3):424-38.
- 25 Chen J, Ivashkiv LB. IFN-gamma abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodeling. *Proceedings of the National Academy of Sciences of the United States of America*. 2010 Nov 9;107(45):19438-43.
- 26 Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol*. 2014 Oct 25;155(2):213-9.
- 27 Uze G, Schreiber G, Piehler J, Pellegrini S. The receptor of the type I interferon family. *Curr Top Microbiol Immunol*. 2007;316:71-95.
- 28 Buro LJ, Chipumuro E, Henriksen MA. Menin and RNF20 recruitment is associated with dynamic histone modifications that regulate signal transducer and activator of transcription 1 (STAT1)-activated transcription of the interferon regulatory factor 1 gene (IRF1). *Epigenetics Chromatin*. 2010;3(1):16.



Supplementary Figure 1: β -glucan-induced trained immunity in CMC patients. PBMCs from healthy volunteers and CMC patients were trained for 24 hours with culture medium or to very low concentrations of β -glucan (1 μ g/ml). At day 6, a second stimulation with LPS (10 ng/ml) was performed for an additional 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls, and $n = 5$ CMC patients. The data are presented as the means \pm SEM. HC: healthy control; CMC: Chronic Mucocutaneous Candidiasis; LPS: Lipopolysaccharide.



Supplementary Figure 2: β -glucan-induced trained immunity in HIES patients. PBMCs from healthy volunteers and HIES patients were trained for 24 hours with culture medium or to very low concentrations of β -glucan (1 μ g/ml). At day 6, a second stimulation with LPS (10 ng/ml) was performed for an additional 24 h. TNF- α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls, and $n = 3$ HIES patients. The data are presented as the means \pm SEM. HC: healthy control; HIES: Hyper-IgE syndrome; LPS: Lipopolysaccharide.

8

Antifungal innate immunity: recognition and inflammatory networks

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Abstract

A large variety of fungi are present in the environment, among which a proportion colonizes the human body, usually without causing any harm. However, depending on the host immune status, commensals can become opportunistic pathogens that induce diseases ranging from superficial non-harmful infection to life-threatening systemic disease. The interplay between the host and the fungal commensal flora is being orchestrated by an efficient recognition of the microorganisms, which in turn ensures a proper balance between tolerance of the normal fungal flora and induction of immune defence mechanisms when invasion occurs. Pattern recognition receptors (PRRs) play a significant role in maintaining this balance due to their capacity to sense fungi and induce host responses such as the induction of proinflammatory cytokines involved in the activation of innate and adaptive immune responses. In the present review we will discuss the most recent findings regarding the recognition of *Candida albicans* and *Aspergillus fumigatus* and the different types of immune cells that play a role in antifungal host defence.

Keywords: *Candida*, *Aspergillus*, pattern recognition receptors, inflammatory networks

Introduction

During the last few decades, the prevalence of fungal infection has considerably increased, especially due to the immunosuppressive treatments in cancer therapy, transplantation, and the HIV epidemics (1). Moreover, pathogenic fungi are able to rapidly adapt and become resistant to antifungal agents (2). The human commensal *Candida albicans* and the environmental fungus *Aspergillus fumigatus* are facultative pathogens, which in immunocompetent individuals do not cause infection. However, immunocompromised patients are prone to systemic fungal infections in which mortality rates can rise up to 60% (3). While the clinical presentation of candidiasis and aspergillosis differs, the mechanisms needed to control infection, including the pattern recognition and inflammatory networks, share many characteristics.

Pattern recognition receptors in fungal infection

Both *Candida* and *Aspergillus* undergo a continuous morphology switch between yeasts and hyphae, resulting in differential exposure of fungal pattern associated molecular patterns (PAMPs). *C. albicans* cell wall contains carbohydrate polymers and glycoproteins, comprising mainly chitin, β -1,3- and β -1,6-glucan and O- and N-linked mannan. The *Aspergillus* cell wall is predominantly composed of chitin, α -glucans, β -(1,3)-(1,4)-glucans, and galactomannans. One important feature of the *Aspergillus* conidial cell wall is the outer layer of hydrophobic rodlets. During swelling of the conidial cell wall and subsequent germination, the hydrophobic layer changes into a hydrophilic structure exposing polysaccharides. During the course of fungal infection and invasion, the first crucial step in mounting effective immune responses is the recognition of the invaders by the host. Various pattern recognition receptors (PRRs) on different cell populations will recognize fungal invaders and trigger bona fide signalling pathways and cellular responses (Figure 1).

C-type lectin receptors

Mannans present in the outer layer of *Candida* and *Aspergillus* cell wall come in direct contact with the host and can be recognized by the C-type lectin receptors (CLRs). Mannose receptor (MR), primarily present on macrophages, recognizes *Candida* N-mannan (4), and further induces IL-17 (5). MR has also been shown to recognize *Aspergillus* conidia and induces proinflammatory cytokines (5). Dectin-2, which is mainly expressed on dendritic cells (DCs), macrophages and neutrophils, recognizes *Candida* α -mannan and also triggers IL-17 (6). In addition to its role in modulating T helper (Th) responses, Dectin-2 has also been associated with ROS production, as well as phagocytosis and killing of *Candida glabrata* (7). Dectin-2 forms heterodimers with Dectin-3, leading to proinflammatory responses upon *C. albicans* infection (8). Dectin-2 is also important for the *Aspergillus*-specific immune response, being up-regulated upon recognition of the fungus. Its expression is restricted to the population of monocyte-derived macrophages (9).

Galectin-3 on macrophages recognizes β -mannans (10), and induces a protective antifungal response in murine macrophages through the secretion of TNF- α . Moreover,

mice deficient in Galectin-3 are more susceptible to disseminated candidiasis (11). Mincle is a CLR expressed on monocytes and neutrophils, yet with an unknown ligand. In murine models, Mincle was responsible for inducing protective responses against *C. albicans*, mainly by the initiation of TNF- α production; mice deficient in this receptor were drastically susceptible to systemic candidiasis (12). However, the role of Mincle in *Aspergillus* recognition is still unknown. DC-SIGN is present on dendritic cells as well as macrophages and recognizes *Candida* N-linked mannan (13), and can bind and internalize *Aspergillus* conidia (14). It induces a cascade of cytokines that activate Th cells in order to differentiate and initiate adaptive immune responses. Mannose-binding lectin (MBL) is a soluble CLR that also binds mannan modulating the recruitment of phagocytes and proinflammatory responses against *Candida* (15).

β -1,3 and β -1,6-glucan is shielded by the mannoproteins in *C. albicans* yeast, but they are exposed on budding yeast, and *Candida* hyphae. Dectin-1, a well-described CLR present on macrophages and monocytes, binds to a long β -1,3-glucan chain (16) inducing cytokines as well as internalization of the fungus upon formation of a “phagocytic synapse” (17). Resting *Aspergillus* conidia do not present abundant β -glucans on their surface either, which might account for the redundant role of Dectin-1 in induction of different Th subsets in experimental *in vitro* models (18, 19). Inhibition of Dectin-1 on alveolar macrophages did not affect phagocytosis of resting *A. fumigatus* conidia (20). In contrast, alveolar macrophages stimulated with germinated conidia produce innate cytokines via the Dectin-1 pathway (21). Dectin-1 was described to be involved in both clinical spectra of aspergillosis: in an invasive aspergillosis mouse model, IL-17 production is mediated by Dectin-1 and was dependent on IL-23 (22), and in an allergic mouse model, Dectin-1 was demonstrated to drive pulmonary pathology via IL-22 production (23). Corticosteroids block *Aspergillus*-induced autophagy via the Dectin-1/Syk pathway (24), and corticosteroid-resistant asthma was associated with detrimental IL-17 and IL-22 production (25), suggesting a strong role for autophagy and Dectin-1 to drive pathology in severe forms of asthma. Dectin-1 polymorphisms were not associated with disseminated candidiasis in humans, but were associated with *Candida* colonization, while susceptibility to invasive aspergillosis was dependent on Dectin-1 polymorphisms (26, 27). In conclusion, CLRs play an important role in mediating immunity against *Candida* and *Aspergillus* through inducing Th-derived cytokines and mediating uptake of the fungus.

8

Toll like receptors

Toll like receptors (TLRs) have been extensively studied in the context of fungal recognition. TLRs play a significant role in a murine model of disseminated candidiasis and, together with IL-1 receptor, activate several potent antifungal-signalling mechanisms (28). TLR2^{-/-} mice were reported to have an increased susceptibility to disseminated candidiasis that could be attributed to a decreased secretion of TNF- α and MIP-2 and impaired recruitment of neutrophils (29). In contrast, others have shown that TLR2^{-/-} mice are more resistant to systemic candidiasis exhibiting an increased chemotaxis and IFN- γ secretion, while IL-10 was significantly impaired. Interestingly, the recruitment of monocytes was enhanced in

TLR2^{-/-} mice, suggesting an increase in the candidacidal activity in these mice (30). TLR4^{-/-} mice are more susceptible to systemic candidiasis due to an impaired secretion of KC and decreased neutrophil recruitment resulting in a higher fungal burden in the kidneys (31). However, TLR1 polymorphisms, but not TLR2 and TLR4 polymorphisms, have been associated with human susceptibility to *C. albicans* infection (27). TLR4^{-/-} mice were more susceptible to *Aspergillus* infections, and TLR4 polymorphisms were associated with invasive aspergillosis (28, 32). Immunosuppression of TLR2^{-/-} mice resulted in higher susceptibility to *Aspergillus* infections (33); however polymorphisms in TLR2 were not associated with invasive aspergillosis (26). The intracellular receptor TLR3 recognizes fungal nucleic acids. TLR3 had a protective role against *Candida*, as a mutation in the L412F variant of TLR3 leads to lower activation of nuclear factor-kappa B (NF-κB) and decreased levels of IFN-γ, resulting in increased susceptibility to cutaneous candidiasis (34). TLR3^{-/-} and TRIF^{-/-} mice were found to be susceptible to invasive pulmonary aspergillosis, and TLR3 polymorphisms have been associated with invasive aspergillosis (26, 35), suggesting an important role of TLR3 in mediating protection against *Aspergillus* infection as well. Conclusions on the role of TLR7 and TLR9 in fungal infection have not been consistent, and no polymorphisms in TLR7 and TLR9 have been associated to date with invasive aspergillosis or candidiasis (26, 27).

NOD-Like receptors

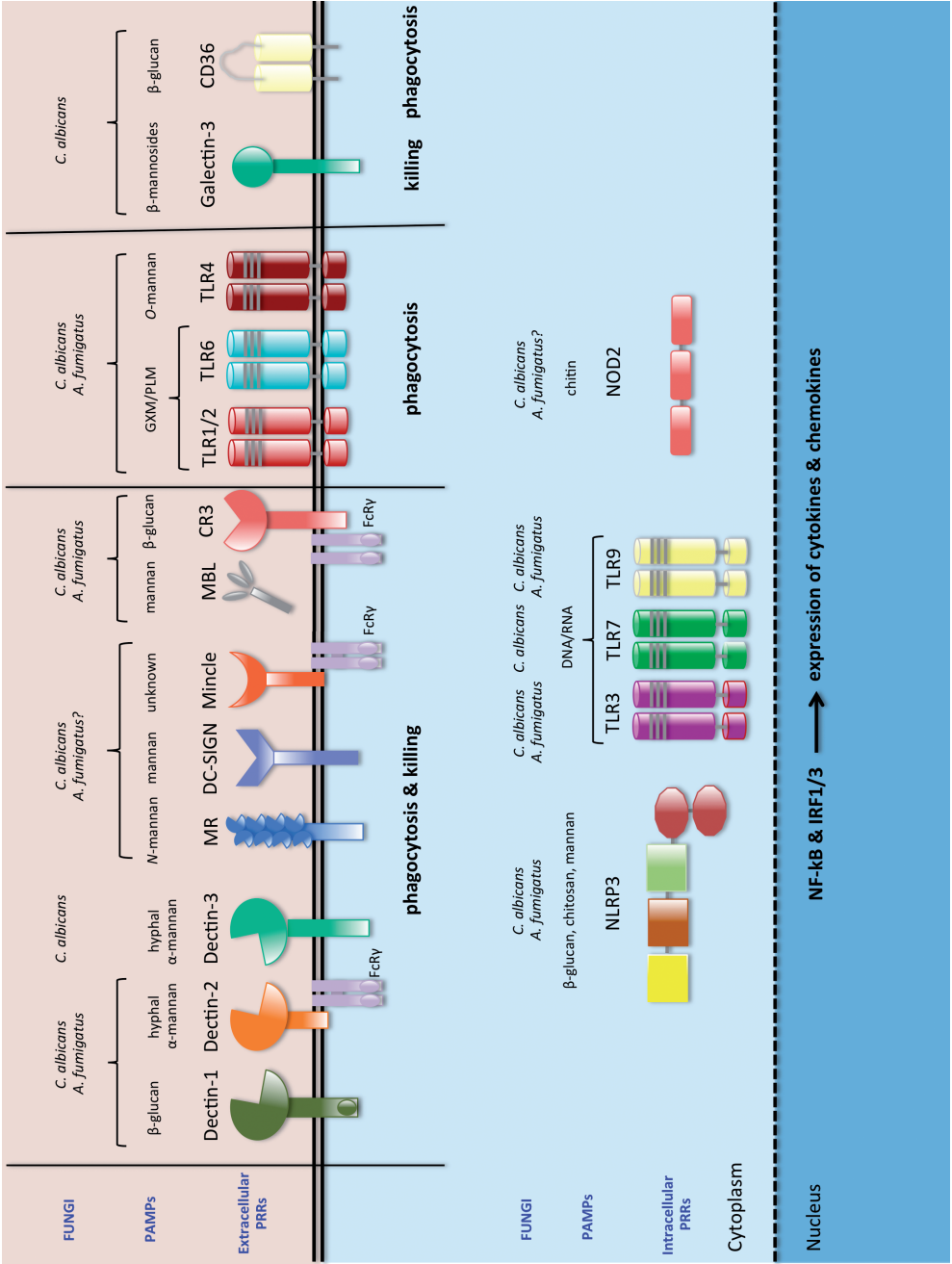
NOD1 and NOD2 are intracellular NOD-Like receptors (NLRs) that recognize bacterial peptidoglycans. Chitin-mediated responses, such as the induction of IL-10, were found to be dependent on NOD2 as well as TLR9 and MR (36). However, there is presently only *in vitro* data to support a role for NOD2 in fungal infection, no *in vivo* studies in NOD2 mice have been performed, and no studies have been performed on the role of NOD1 in fungal infection. The best-characterized NLR is NLRP3, which is a component of the inflammasome. Mice deficient in NLRP3 are more susceptible to *Candida* infections (37-39). One study showed involvement of NLRP3 in anti-*Aspergillus* host defence *in vitro*; however, disseminated aspergillosis has not been studied in NLRP3-deficient mice (40).

Inflammatory networks

Recognition of fungal-associated molecular patterns by PRRs will lead to the initiation of an efficient host defence that is mandatory to clear the invading fungus. Several populations of cells are involved that orchestrate this clearance (Figure 2).

Epithelial cells

The epithelium constitutes a mechanical barrier against invading fungi. *C. albicans* is in permanent contact with epithelial cells and infection with tissue invasion is associated with morphological changes of yeast into hyphae. *Candida* hyphae can penetrate the epithelial cells through two distinct mechanisms: induced endocytosis and active penetration.



- ◀ Fig. 1: Recognition of *C. albicans* and *A. fumigatus* by innate immune cells. The pattern recognition receptors Dectin-1, Dectin-2, Dectin-3, FC gamma receptor (FcRγ), Mannose Receptor (MR), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN), Mincle, Mannan-binding lectin (MBL), Complement Receptor 3 (CR3), Galectin-3, Cluster of Differentiation 36 (CD36), Toll-like receptors (TLRs), NACHT, LRR, and PYD domain-containing protein 3 (NLRP3), and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) recognize the fungal cell wall components β-glucan, α-mannan, β-mannan, N/O-mannan, phospholipo-mannan (PLM), glucuronoxylomannan (GXM), chitosan, chitin, or fungal DNA and RNA. These signals activate canonical or non-canonical nuclear pathways, with the subsequent induction of an immune response, such as secretion of cytokines, chemokines, initiation of phagocytosis or inflammasome activation.

Epithelial cells respond to *Candida* with activation of NF-κB and cytokine production when two pathways are induced; the second pathway, namely MPK1 and c-Fos activation, was dependent on hyphal formation. In this way, conidia are tolerated and germination is seen as a danger signal in which the epithelium plays an important initial step to induce a protective inflammatory network (41). *Aspergillus* species are not typical commensals. Lung epithelium not only acts as an efficient barrier, it can also phagocytose *Aspergillus* conidia. Although primary epithelial cells from the respiratory tract phagocytose *Aspergillus* conidia, the killing capacity of these cells is low (42). However, they can control outgrowth, and in addition potent antifungal drugs such as posocanazol, can accumulate in the membranes of lung epithelial cells and, in this way, might contribute to efficient exposure of antifungal drugs during *Aspergillus* infection. Thus, epithelial cells can play an important role in fungal infection by restricting fungal outgrowth and initiate potent antifungal host responses.

Monocytes and macrophages

Residential macrophages are phagocytes that are essential in mediating the first steps of an effective antifungal host defence. Fungi have evolved strategies to escape these initial first steps. *Candida* can inhibit phagolysosome maturation and production of NO by macrophages, while *Aspergillus* can inhibit phagolysosome acidification (20). In addition, fungi can change the characteristics of macrophages; *Candida* infection can induce a more anti-inflammatory phenotype enhancing fungal survival by switching M1 macrophages to a M2 phenotype (43). Recently, the importance of residential macrophages to control disseminated fungal infection was elegantly demonstrated. A deficiency in accumulation of residential macrophages in the kidney due to a deficiency in the chemokine receptor CX3CR1 lead to renal failure and death. Moreover, patients with a polymorphism leading to a decreased function in CX3CR1 were more susceptible to disseminated candidiasis. These data highlight the essential role of residential macrophages, in particular, in the kidney to control disseminated fungal infection (44). In addition to macrophages, monocytes can also phagocytose spores and inhibit germination. Monocytes have a potent conidial killing capacity and are essential in the first 48h to control disseminated candidiasis (45). Moreover, monocytes can rapidly differentiate into DC that transport spores to draining lymph nodes, where they prime and expand *A. fumigatus*-specific Th cells (46). In addition, monocytes can augment neutrophil conidiacidal activity and play a crucial role in controlling disseminated fungal infection (47).

Neutrophils

Neutrophils are essential to clear fungal invasion, which is underscored by the observation that immunosuppression with prolonged neutropenia is a major risk factor for invasive fungal infections. Various neutropenic mouse models have clearly demonstrated a significant role for neutrophils in disseminated fungal infection. Mice depleted of alveolar macrophages prior to pulmonary aspergillosis recruited neutrophils normally and are able to restrict hyphal tissue invasion, while neutrophil depletion prior to or within 3 h after infection resulted in high susceptibility (48). Neutrophils use oxidative and non-oxidative killing mechanisms, which are both essential in *Candida* and *Aspergillus* infections. The oxidative burst is produced by the enzymes NADPH oxidase and myeloperoxidase. NADPH oxidase deficiency results in the disease chronic granulomatous disease (CGD), which is associated with an extraordinary increased susceptibility to invasive mold infection, highlighting the importance of NADPH-oxidase in controlling fungal infection. Another specific feature of neutrophils is their capacity to rapidly release granules that mediate extracellular killing. Those granules contain, next to NADPH oxidase and myeloperoxidase, other proteins with antimicrobial properties, like lysozymes, lactoferrins, elastases, defensins, gelatinases or cathepsin G (49). In a cell-free supernatant of degranulated neutrophils of CGD patients, a normal amount of iron-free lactoferrin was found, which was capable to decrease fungal growth (50). Additionally, neutrophil elastase and cathepsin B were described to have antifungal activity and elastase was found to contribute to neutrophil extracellular traps (NETs) (51). NETs, which are composed of DNA that forms large fibrillar structures that can bind pathogens and antimicrobial peptides, provide a mechanism to combat hyphae that are too big to be phagocytosed. It was demonstrated that *Candida* can be captured by NETs, however it did not contribute to efficient killing (52). Similarly, NETs were able to limit fungal growth, but not to kill *Aspergillus* conidia and hyphae (51, 53). Bianchi *et al.* have demonstrated that NET formation is deficient in CGD patients and that NET formation can be restored by gene-therapy resulting in successful neutrophil-dependent elimination of *A. nidulans* conidia and hyphae. The neutrophil-associated calprotectin was identified to inhibit growth of *A. nidulans* in the NETs of CGD mice after gene therapy (54). The antifungal activity of calprotectin is not restricted to *Aspergillus*; it was also identified as the main component in NETs controlling *Candida* growth *in vivo* and *in vitro* (55). CGD is not only characterised by a deficiency in neutrophil killing and NET formation. The NADPH oxidase is also present in monocytes and macrophages, and has been shown to be also important for controlling fungal infection (56).

In addition, macrophages and monocytes deficient in NADPH-oxidase have a defect in autophagy, an intracellular mechanism that can control *Aspergillus* outgrowth (57). Whereas autophagy appears to be crucial for *Aspergillus* infection, it seems redundant in candidiasis, although initially it was reported that mice deficient in autophagy were more susceptible to *Candida* infection (58, 59). A selective killing defect by neutrophils for *C. albicans*, but not for *A. fumigatus*, was found in CARD9 deficient neutrophils resulted in abnormal ultrastructural phagolysosomes and massive hyphal outgrowth, which was independent of ROS production and release of azurophilic granules. Neutrophils were found to display two independent killing

mechanisms: ROS-dependent that is crucial for clearing opsonized *Candida* and CARD9 dependent that is crucial for unopsonized *Candida* (60). This suggests that neutrophils have an unknown killing mechanism that is specific for *Candida* initiated by CARD9.

IL-17 is a potent mediator of neutrophil recruitment. Next to Th cells and innate lymphoid cells, neutrophils themselves have been reported as a source for IL-17, in a Dectin-1- and IL-23-dependent manner, and ROR γ T-positive neutrophils exposed to IL-23 and IL-6 express the receptor IL-17RC (61). This suggests an autocrine feedback loop in recruiting neutrophils to the site of the fungal infection. Mice deficient in IL-17 signalling are more susceptible to systemic candidiasis. However, patients with an IL-17 deficiency suffer from mucosal candidiasis, but do not develop invasive candidiasis, and are not more susceptible to invasive mould infection. It is still an open question to what extent IL-17 determines neutrophil faith and function at the mucosa. In a mouse model of oropharyngeal candidiasis, IL-17RA^{-/-} and IL-23^{-/-} mice depleted for neutrophils show higher fungal burden compared to non-neutropenic animals, suggesting that IL-17 deficiency does not result in a complete neutrophil function at the site of infection (62). In addition, the recruitment of neutrophils by vaginal epithelial cells producing S100 alarmins was independent of IL-17 production (63).

Dendritic cells

Dendritic cells (DCs) achieve a crucial patrolling function. They are located in the mucosa and migrate to the draining lymph nodes to activate T cells. DCs stimulated with the fungal pathogens *Aspergillus* and *Candida* show increased activation markers like CD80 and CD86, as well as IL-8 and IL-12p70 production (64). Moreover, DCs play a crucial role for the host response against *Candida* via the production of type-I IFN- β in a Dectin/Syk/IRF5 dependent pathway (65–67). DCs are mainly involved in activation and shaping Th responses. During pulmonary *Aspergillus* infection conidial transport from the lung to the lung-draining lymph nodes, priming of CD4⁺ T cell responses and the prevention of the pulmonary fungal burden was mediated by monocyte-derived CD11b⁺ DC population in a murine model (46). *Aspergillus* conidia triggered protective Th1 and Treg responses, whereas *Aspergillus* hyphae triggered an anti-inflammatory Th2/Th17 profile (68). The protective DC responses are dependent on the PI3K/Akt/mTOR pathway, while the anti-inflammatory DCs depend on STAT3/IDO activation (68). The direction of the airway inflammation in mice into either Th2 or Th17 was dependent on TNF- α produced by monocyte-derived DCs: BALB/c mice showed a high amount of TNF- α -producing DCs promoting Th17 responses with subsequent neutrophils recruitment, while C57BL/6 mice displayed a low amount of TNF- α -producing DCs, associated with high IL-5 levels and eosinophilia (69). Plasmacytoid DCs (pDCs) represent a specific subset of DCs, and, in the context of fungal infections, have not been studied in great extent. In a mouse model of invasive aspergillosis a specific influx of pDCs was observed, and depletion of pDCs resulted in an increased susceptibility to invasive aspergillosis. *In vitro* analysis showed that hyphal growth was inhibited by pDCs in a contact-independent manner. Interestingly, pDCs produced inflammatory cytokines, such as IFN- α and TNF- α , that were independent of TLR9 (70). In contrast, pDCs in a vaginal candidiasis model were suggested to

mediate tolerance, since pDCs that were present in the draining lymph nodes of the infection lacked the upregulation major histocompatibility complex class II, CD80, CD86, and CD40 (71). This could point to a crucial difference in pDC responses induced by *Aspergillus* or *Candida* that might be dependent on the site of infection.

Innate lymphoid cells

Recently, a novel population of innate lymphocytes called innate lymphoid cells (ILCs) has been identified. ILCs are analogous to Th subsets, but differ because they lack the T cell receptor. They have been named according to their cytokine profile. ILC1 cells express T-bet and produce IFN- γ , ILC2 cells express GATA3 and produce IL-5 and IL-13, and ILC3 cells express ROR γ t and produce IL-17 and IL-22. Only a few studies so far have deciphered a role for innate lymphoid cells in the host defence against *Aspergillus* or *Candida*. The IL-17-producing ILC3 cells have been described as being important in the defence against and the control of pathogens at the mucosal barrier. *C. albicans* is a potent inducer of IL-17 (72) and mice lacking IL-17 are highly susceptible to *Candida* infections (73). As described earlier, patients with an IL-17 deficient phenotype such as hyper IgE syndrome (74) and patients with chronic mucocutaneous candidiasis (75), suffer from mucosal candidiasis, but the lack of IL-17 in these patients has been predominantly attributed to Th17 cells. Innate lymphoid cells would supply a faster source of IL-17 than the adaptive Th17 cells, and they represent an additional important source of IL-17.

The first clues that ILCs play an important role in controlling fungal infection and colonization come from experimental mouse models. Fungal control in a murine model of oral candidiasis was mediated by IL-17 secreting ILCs and both, ILC depleted RAG^{-/-} and RORC^{-/-} ILC^{-/-} mice completely failed to control fungal outgrowth (76). The relevance of ILCs in mediating pulmonary host responses in the lung was demonstrated in a study where *Candida* airway exposure was able to protect against *Pseudomonas aeruginosa*-induced lung injury, an effect that was dependent on pulmonary ILCs that expressed IL-22 (77). It has been demonstrated that ILCs play a role in the pathogenesis of asthma and allergic reactions, and the contribution of GATA3⁺ ILCs to allergic reactions mediated by *Aspergillus* has recently been studied in patients with chronic sinusitis. These patients showed a significant proliferation of GATA3⁺ ILCs and increased GATA3⁺ ILCs responsiveness to IL-33 induced by *Aspergillus* extract which resulted in an increased IL-13 production (78). Interestingly, also invariant NKT (iNKT) cells are found to contribute to airway hyperactivity in an IL-33 dependent manner, directly activated by the *Aspergillus* glycosphingolipid Asperamide B (79). This might explain one mechanism via which *Aspergillus* can directly induce allergic responses in an immunocompetent host, and suggests a critical role for ILCs in fungal-induced allergic reactions. The role of ILCs in an immunosuppressive status during invasive fungal infection still needs to be investigated.

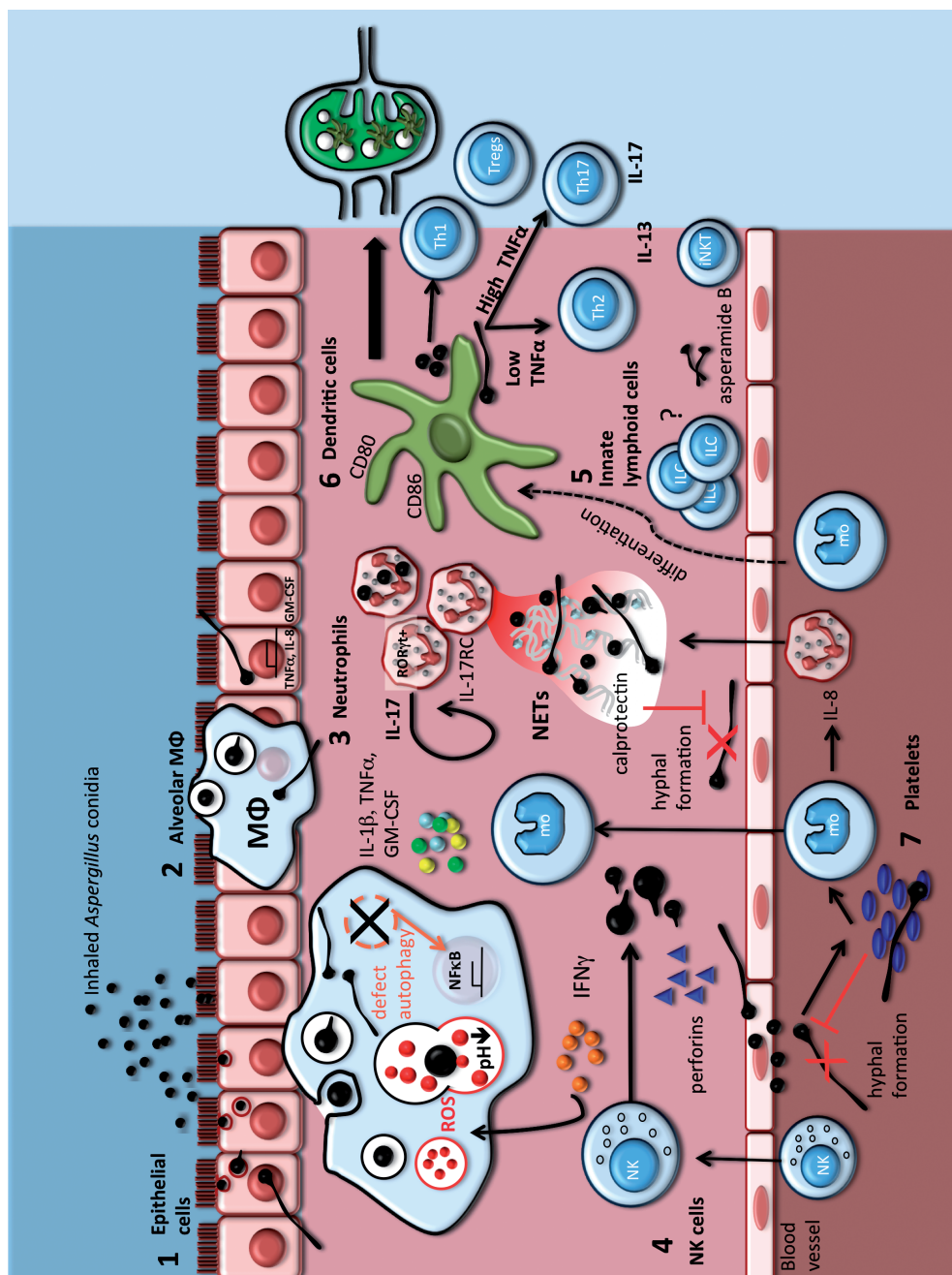
Natural Killer Cells

Natural Killer (NK) cells contribute to the rapid innate immune response against invading pathogens in an antigen-independent manner, mainly by recognizing infected cells and

induce cell death of the infected cell. NK cells also contribute to the host defence against fungal infections. The additional depletion of NK cells in a T/B-cell-deficient SCID mice increases the susceptibility to systemic candidiasis, while it had no (or even an opposite) effect in immunocompetent mice (80). NK cells might substitute for specific functions of B and T cells when these cells are absent, which is in line with the observation that NK cell activation by *Candida* results in the production of high amounts of GM-CSF, IFN- γ and TNF- α , cytokines that are also produced by T cells. Although phagocytosis by NK cells did not inhibit hyphal growth of *Candida*, antifungal activity against *Candida* was observed that was mediated by extracellular perforin production by NK cells (81). Comparably, NK cells could kill *Aspergillus* hyphae via a perforin-mediated mechanism, while *Aspergillus* conidia were not affected (82). Bouzani *et al.* also showed that only germinated morphological forms of *Aspergillus* were able to stimulate NK cells to produce high amount of IFN- γ . However, in contrast to Voigt *et al.* and Schmidt *et al.*, they propose a perforin-independent killing mechanism, since they observed that the killing was mediated via a soluble factor (83). NK cells are potent producers of IFN- γ , and IFN- γ is crucial for controlling *Aspergillus* infection, which is highlighted by the fact that IFN- γ knockout mice display an increased susceptibility to invasive aspergillosis. In a murine model of invasive aspergillosis in which neutrophils were depleted, NK cells were the major population of cells that were able to secrete IFN- γ and to control fungal growth in the lung during early infection. The depletion of NK cells in IFN- γ -deficient mice did not result in increased severity of the infection, while depletion of NK cells in wild type mice resulted in a similar increases susceptibility to infection that was observed in IFN- γ -deficient mice. These data strongly suggest that in neutropenic conditions the protective IFN- γ responses during early invasive aspergillosis are critically dependent on NK cells. (84).

Platelets

Classically, platelets, mediate blood coagulation after interruption of the endothelium. In addition, platelets are capable to mediate innate immune functions, especially during bloodstream infections. Although *in vitro* studies failed to show the capacity of *C. albicans* to aggregate human platelets, *in vivo* platelets bound to *C. albicans* and were activated after bloodstream injection in a murine model (85). Platelets produced peptides like Rantes or platelet factor 4 with antimicrobial activity against *Candida* and platelet-rich plasma inhibited the growth of *Candida* (86). In *Aspergillus* infection, the production of the proinflammatory IL-8 from human monocytes is enhanced by platelets activated by *Aspergillus* hyphae, and all *Aspergillus* morphotypes had the capacity to activate platelets (87). This seems to be an interesting feedback-loop, since *Aspergillus* germination and hyphal formation was inhibited by activated platelets (88). Interestingly, not only the direct contact of the platelets to *Aspergillus* was necessary, also *Aspergillus* cell culture supernatant was able to activate platelets, suggesting a potentially harmful contribution to systemic infection by increasing thrombotic status during invasive aspergillosis (88).



- ◀ Fig. 2: Signalling networks of the innate immune response against *Aspergillus*. (1) Inhaled *Aspergillus* conidia reaching the alveoli are endocytosed by the lung epithelial cells, in which they survive or escape from via hyphal formation. Epithelial cells react upon fungal growth with expression of pro-inflammatory cytokines. (2) Once internalized in alveolar macrophages and monocytes, *Aspergillus* conidia are phagocytosed, which is prevented by hyphal formation. Acidification in the phagolysosomes is essential for the killing of *A. fumigatus* conidia. Reactive oxygen species (ROS) are produced to effectively kill and eradicate *Aspergillus*. A defect ROS system of macrophages and monocytes like in CGD is associated with defect autophagy resulting in pro-inflammatory cytokine production. (3) Upon invasion, neutrophils are recruited by proinflammatory cytokines produced by macrophages and monocytes or by IL-17 expressed in ROR γ t+ neutrophils and acting on the IL-17RC receptor via an autocrine feedback loop. *Aspergillus* conidia are intracellularly killed by the ROS system, while *Aspergillus* conidia and hyphae bind to neutrophils extracellular traps (NETs), which mainly consist of neutrophil DNA and antimicrobial proteins like calprotectin, inhibiting the fungal growth without killing. (4) Natural killer cells are activated by the germinated and hyphal forms of *Aspergillus* and mediate the killing via perforin release or directly via IFN- γ . Next to that, IFN- γ enhances conidial killing by macrophages. (5) The role of innate lymphoid cells (ILCs) in the lung during invasive aspergillosis is largely unknown. Invariant (i) NKT cells are directly activated by asperamide B derived from *Aspergillus* and cause together with IL-13, derived from Th2 cells, allergic airway responses. (6) Monocyte-derived dendritic cells recognize *Aspergillus* conidia and hyphae resulting in upregulation of CD80+ and CD86+ and the production of IL-8 and IL-12p70. DCs shape adaptive Th responses after migration to the lymph node. *Aspergillus* conidia induce preferentially Th1/Treg responses and *Aspergillus* hyphae Th17/Th2 responses. DCs, high positive for TNF, mediate differentiation into the Th17 direction with production of IL-17 and low positive for TNF into the Th2 direction with production of IL-13. (7) As the last step of invasion, *Aspergillus* can overcome the endothelium and reach the blood stream. All growth forms of *Aspergillus* activate platelets, which activate monocytes to produce IL-8. Activated platelets are capable of inhibiting hyphal formation.

Conclusions and future perspectives

During the past years, fungal pathogens have been of rising interest especially due to the increasing number of immunocompromised patients. In order to combat fungal infections, the human immune system needs to be able to efficiently sense the fungal pathogen, and to activate a plethora of immune cells that are able to engulf and clear the pathogen. In this regard, PRRs are of significant importance. Numerous studies revealed beneficial as well as detrimental roles of different TLRs in fungal infections. Furthermore, several studies on murine models are contradictory, probably due to the differences in the experimental setup, mouse strains that are used, as well as differences in fungal strains. Although many PRRs that recognize fungal PAMPs have been identified, there is still a large amount of unexplored PRRs that could contribute to antifungal host defence. Moreover, the interactions that have been described between PRRs point to an even more complex dynamic cell wall recognition system on immune cells. The knowledge that there could be many fungal PRRs that are dynamically expressed and have the capacity to interact with each other indicates that understanding fungal recognition at the host cell membrane needs further studies and will be challenging and complex.

Fungal recognition as well as the interplay between the innate immune cells forms the initial inflammatory network that is needed to control fungal infection. A better understanding of the role of epithelial cells in fungal infection has led to an increased awareness that non-classical immune cells, such as epithelial cells, endothelial cells, and platelets, also play a crucial role in the orchestration of an efficient antifungal host response. In addition, new subsets of immune cells, such as ILCs have come to stage and NK cells have gained interest in their contribution to control fungal infection. These new players next to

the traditional and well-known professional phagocytes such as neutrophils and monocyte/macrophages open up relevant new questions. To what extent does immunosuppression with corticosteroids affect these new players? And in turn, does this influence the function of the classical phagocytes? Does NADPH-oxidase deficiency influence effective epithelial and endothelial antifungal host responses? In addition, recent studies have suggested an innate immune memory in NK cells as well as in monocytes, which opens new horizons regarding the host-fungus interactions. Hence, not only CLRs, but also some TLRs were shown to mediate immune responses to a microbe, upon a second encounter, by either strengthening or diminishing host responses by the means of host chromatin remodelling that can shape the host immune response (89, 90), making these receptors interesting candidates for further investigation as potential targets for vaccines.

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References

- 1 Yapar N. Epidemiology and risk factors for invasive candidiasis. Therapeutics and clinical risk management. [Review]. 2014;10:95-105.
- 2 Vermeulen E, Lagrou K, Verweij PE. Azole resistance in *Aspergillus fumigatus*: a growing public health concern. Current opinion in infectious diseases. [Research Support, Non-U.S. Gov't Review]. 2013 Dec;26(6):493-500.
- 4 Sudbery P, Gow N, Berman J. The distinct morphogenic states of *Candida albicans*. Trends in microbiology. 2004 Jul;12(7):317-24.
- 5 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol. 2008 Jan;6(1):67-78.
- 6 Chai LY, van de Veerdonk F, Marijnissen RJ, Cheng SC, Khoo AL, Hectors M, et al. Anti-*Aspergillus* human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. Immunology. [Research Support, Non-U.S. Gov't]. 2010 May;130(1):46-54.
- 7 Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity. 2010 May 28;32(5):681-91.
- 8 Ifrim DC, Bain JM, Reid DM, Oosting M, Verschuere I, Gow NA, et al. Role of Dectin-2 for host defense against systemic infection with *Candida glabrata*. Infect Immun. 2014 Mar;82(3):1064-73.
- 9 Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, et al. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. Immunity. 2013 Aug 22;39(2):324-34.
- 10 Sun H, Xu XY, Shao HT, Su X, Wu XD, Wang Q, et al. Dectin-2 is predominately macrophage restricted and exhibits conspicuous expression during *Aspergillus fumigatus* invasion in human lung. Cell Immunol. [Research Support, Non-U.S. Gov't]. 2013 Jul-Aug;284(1-2):60-7.
- 11 Jouault T, El Abed-El Behi M, Martinez-Esparza M, Breuilh L, Trinel PA, Chamaillard M, et al. Specific recognition of *Candida albicans* by macrophages requires galectin-3 to discriminate *Saccharomyces cerevisiae* and needs association with TLR2 for signaling. J Immunol. 2006 Oct 1;177(7):4679-87.
- 12 Linden JR, De Paepe ME, Laforce-Nesbitt SS, Bliss JM. Galectin-3 plays an important role in protection against disseminated candidiasis. Med Mycol. 2013 Aug;51(6):641-51.
- 13 Wells CA, Salvage-Jones JA, Li X, Hitchens K, Butcher S, Murray RZ, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. J Immunol. 2008 Jun 1;180(11):7404-13.
- 14 Cambi A, Netea MG, Mora-Montes HM, Gow NA, Hato SV, Lowman DW, et al. Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. J Biol Chem. 2008 Jul 18;283(29):20590-9.
- 15 Serrano-Gomez D, Dominguez-Soto A, Ancochea J, Jimenez-Heffernan JA, Leal JA, Corbi AL. Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin mediates binding and internalization of *Aspergillus fumigatus* conidia by dendritic cells and macrophages. J Immunol. 2004 Nov 1;173(9):5635-43.
- 16 Saevarsdottir S, Vikingsdottir T, Valdimarsson H. The potential role of mannan-binding lectin in the clearance of self-components including immune complexes. Scand J Immunol. 2004 Jul-Aug;60(1-2):23-9.
- 17 Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, et al. Dectin-1 is a major beta-glucan receptor on macrophages. J Exp Med. 2002 Aug 5;196(3):407-12.
- 18 Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, et al. Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature. 2011 Apr 28;472(7344):471-5.
- 19 Gresnigt MS, Becker KL, Smeekens SP, Jacobs CW, Joosten LA, van der Meer JW, et al. *Aspergillus fumigatus*-induced IL-22 is not restricted to a specific Th cell subset and is dependent on complement receptor 3. J Immunol. 2013 Jun 1;190(11):5629-39.
- 20 Becker KL, Gresnigt MS, Smeekens SP, Jacobs CW, Magis-Escarra C, Jaeger M, et al. Pattern recognition pathways leading to a Th2 cytokine bias in ABPA patients. Clin Exp Allergy. 2014 Jun 10.
- 21 Slesiona S, Gressler M, Mihlan M, Zaehle C, Schaller M, Barz D, et al. Persistence versus escape: *Aspergillus terreus* and *Aspergillus fumigatus*

- employ different strategies during interactions with macrophages. *PLoS One*. [Research Support, Non-U.S. Gov't]. 2012;7(2):e31223.
- 22 The Beta-Glucan Receptor Dectin-1 Recognizes Specific Morphologies of *Aspergillus fumigatus*, (2005).
 - 23 Werner JL, Gessner MA, Lilly LM, Nelson MP, Metz AE, Horn D, et al. Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infect Immun*. [Research Support, N.I.H., Extramural]. 2011 Oct;79(10):3966-77.
 - 24 Lilly LM, Gessner MA, Dunaway CW, Metz AE, Schwiebert L, Weaver CT, et al. The beta-glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J Immunol*. [Research Support, N.I.H., Extramural]. 2012 Oct 1;189(7):3653-60.
 - 25 Kyrmizi I, Gresnigt MS, Akoumianaki T, Samonis G, Sidiropoulos P, Boumpas D, et al. Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting dectin-1/Syk kinase signaling. *J Immunol*. [Research Support, N.I.H., Extramural]
 - 26 Research Support, Non-U.S. Gov't]. 2013 Aug 1;191(3):1287-99.
 - 27 McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol*. [Research Support, N.I.H., Extramural]. 2008 Sep 15;181(6):4089-97.
 - 28 Cunha C, Aversa F, Romani L, Carvalho A. Human genetic susceptibility to invasive aspergillosis. *PLoS pathogens*. 2013;9(8):e1003434.
 - 29 Smeeckens SP, van de Veerndonk FL, Kullberg BJ, Netea MG. Genetic susceptibility to *Candida* infections. *EMBO molecular medicine*. 2013 Jun;5(6):805-13.
 - 30 Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, et al. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol*. [Research Support, Non-U.S. Gov't]
 - 31 Research Support, U.S. Gov't, P.H.S.]. 2004 Mar 1;172(5):3059-69.
 - 32 Villamon E, Gozalbo D, Roig P, O'Connor JE, Ferrandiz ML, Fradelizi D, et al. Toll-like receptor 2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model of disseminated candidiasis. *Microbes Infect*. 2004 May;6(6):542-8.
 - 33 Netea MG, Suttmuller R, Hermann C, Van der Graaf CA, Van der Meer JW, van Krieken JH, et al. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol*. 2004 Mar 15;172(6):3712-8.
 - 34 Netea MG, Van Der Graaf CA, Vonk AG, Verschuere I, Van Der Meer JW, Kullberg BJ. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis*. 2002 May 15;185(10):1483-9.
 - 35 Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, et al. Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *The New England journal of medicine*. 2008 Oct 23;359(17):1766-77.
 - 36 Balloy V, Si-Tahar M, Takeuchi O, Philippe B, Nahori MA, Tanguy M, et al. Involvement of toll-like receptor 2 in experimental invasive pulmonary aspergillosis. *Infect Immun*. [Research Support, Non-U.S. Gov't]. 2005 Sep;73(9):5420-5.
 - 37 Nahum A, Dadi H, Bates A, Roifman CM. The L412F variant of Toll-like receptor 3 (TLR3) is associated with cutaneous candidiasis, increased susceptibility to cytomegalovirus, and autoimmunity. *J Allergy Clin Immunol*. 2011 Feb;127(2):528-31.
 - 38 Carvalho A, De Luca A, Bozza S, Cunha C, D'Angelo C, Moretti S, et al. TLR3 essentially promotes protective class I-restricted memory CD8(+) T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. *Blood*. 2012 Jan 26;119(4):967-77.
 - 39 Wagener J, Malireddi RK, Lenardon MD, Koberle M, Vautier S, MacCallum DM, et al. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog*. 2014 Apr;10(4):e1004050.
 - 40 Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med*. [Research Support, Non-U.S. Gov't]. 2009 Aug 31;206(9):2037-51.
 - 41 Gross O, Poeck H, Bscheidt M, Dostert C, Hanneschlager N, Endres S, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. [Research Support, Non-U.S. Gov't]. 2009 May 21;459(7245):433-6.
 - 42 Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, et al. An essential role for the

- NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. *Cell Host Microbe*. [Research Support, N.I.H., Extramural]. 2009 May 8;5(5):487-97.
- 43 Said-Sadier N, Padilla E, Langsley G, Ojcius DM. *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS One*. [Research Support, Non-U.S. Gov't]. 2010;5(4):e10008.
 - 44 Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. *Cell Host Microbe*. 2010 Sep 16;8(3):225-35.
 - 45 Botterel F, Gross K, Ibrahim-Granet O, Khoufache K, Escabasse V, Coste A, et al. Phagocytosis of *Aspergillus fumigatus* conidia by primary nasal epithelial cells in vitro. *BMC Microbiol*. 2008;8:97.
 - 46 Reales-Calderon JA, Aguilera-Montilla N, Corbi AL, Molero G, Gil C. Proteomic characterization of human proinflammatory M1 and anti-inflammatory M2 macrophages and their response to *Candida albicans*. *Proteomics*. 2014 Jun;14(12):1503-18.
 - 47 Lionakis MS, Swamydas M, Fischer BG, Plantinga TS, Johnson MD, Jaeger M, et al. CX3CR1-dependent renal macrophage survival promotes *Candida* control and host survival. *The Journal of clinical investigation*. 2013 Dec 2;123(12):5035-51.
 - 48 Ngo LY, Kasahara S, Kumasaka DK, Knoblaugh SE, Jhingran A, Hohl TM. Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. *The Journal of infectious diseases*. 2014 Jan 1;209(1):109-19.
 - 49 Hohl TM, Rivera A, Lipuma L, Gallegos A, Shi C, Mack M, et al. Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe*. 2009 Nov 19;6(5):470-81.
 - 50 Espinosa V, Jhingran A, Dutta O, Kasahara S, Donnelly R, Du P, et al. Inflammatory monocytes orchestrate innate antifungal immunity in the lung. *PLoS pathogens*. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. 2014 Feb;10(2):e1003940.
 - 51 Mircescu MM, Lipuma L, van Rooijen N, Pamer EG, Hohl TM. Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. *The Journal of infectious diseases*. [Research Support, N.I.H., Extramural]. 2009 Aug 15;200(4):647-56.
 - 52 Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. 2012;30:459-89.
 - 53 Zarembek KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin JI. Human polymorphonuclear leukocytes inhibit *Aspergillus fumigatus* conidial growth by lactoferrin-mediated iron depletion. *J Immunol*. 2007 May 15;178(10):6367-73.
 - 54 McCormick A, Heesemann L, Wagener J, Marcos V, Hartl D, Loeffler J, et al. NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect*. 2010 Nov;12(12-13):928-36.
 - 55 Menegazzi R, Decleva E, Dri P. Killing by neutrophil extracellular traps: fact or folklore? *Blood*. 2012 Feb 2;119(5):1214-6.
 - 56 Bruns S, Kniemeyer O, Hasenberg M, Aimaniananda V, Nietzsche S, Thywissen A, et al. Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog*. 2010 Apr;6(4):e1000873.
 - 57 Bianchi M, Niemiec MJ, Siler U, Urban CF, Reichenbach J. Restoration of anti-*Aspergillus* defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent. *The Journal of allergy and clinical immunology*. [Research Support, Non-U.S. Gov't]. 2011 May;127(5):1243-52 e7.
 - 58 Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog*. 2009 Oct;5(10):e1000639.
 - 59 Grimm MJ, Vethanayagam RR, Almyroudis NG, Dennis CG, Khan AN, D'Auria AC, et al. Monocyte- and macrophage-targeted NADPH oxidase mediates antifungal host defense and regulation of acute inflammation in mice. *J Immunol*. 2013 Apr 15;190(8):4175-84.
 - 60 de Luca A, Smeeckens SP, Casagrande A, Iannitti R, Conway KL, Gresnigt MS, et al. IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans. *Proc Natl Acad Sci U S A*. 2014 Mar 4;111(9):3526-31.
 - 61 Smeeckens SP, Malireddi RK, Plantinga TS, Buffen K, Oosting M, Joosten LA, et al. Autophagy is redundant for the host defense against

- systemic *Candida albicans* infections. *Eur J Clin Microbiol Infect Dis*. 2014 May;33(5):711-22.
- 62 Nicola AM, Albuquerque P, Martinez LR, Dal-Rosso RA, Saylor C, De Jesus M, et al. Macrophage autophagy in immunity to *Cryptococcus neoformans* and *Candida albicans*. *Infection and immunity*. 2012 Sep;80(9):3065-76.
 - 63 Gazendam RP, van Hamme JL, Tool AT, van Houdt M, Verkuijlen PJ, Herbst M, et al. Two independent killing mechanisms of *Candida albicans* by human neutrophils: evidence from innate immunity defects. *Blood*. 2014 Jun 19.
 - 64 Taylor PR, Roy S, Leal SM, Jr., Sun Y, Howell SJ, Cobb BA, et al. Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2. *Nat Immunol*. 2014 Feb;15(2):143-51.
 - 65 Huppler AR, Conti HR, Hernandez-Santos N, Darville T, Biswas PS, Gaffen SL. Role of neutrophils in IL-17-dependent immunity to mucosal candidiasis. *J Immunol*. 2014 Feb 15;192(4):1745-52.
 - 66 Yano J, Kolls JK, Happel KI, Wormley F, Wozniak KL, Fidel PL, Jr. The acute neutrophil response mediated by S100 alarmins during vaginal *Candida* infections is independent of the Th17-pathway. *PLoS One*. 2012;7(9):e46311.
 - 67 Fidan I, Kalkanci A, Yesilyurt E, Erdal B. In vitro effects of *Candida albicans* and *Aspergillus fumigatus* on dendritic cells and the role of beta glucan in this effect. *Adv Clin Exp Med*. 2014 Jan-Feb;23(1):17-24.
 - 68 Biondo C, Signorino G, Costa A, Midiri A, Gerace E, Galbo R, et al. Recognition of yeast nucleic acids triggers a host-protective type I interferon response. *Eur J Immunol*. 2011 Jul;41(7):1969-79.
 - 69 del Fresno C, Soulat D, Roth S, Blazek K, Udalova I, Sancho D, et al. Interferon-beta production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to *C. albicans*. *Immunity*. 2013 Jun 27;38(6):1176-86.
 - 70 Bourgeois C, Majer O, Frohner IE, Lesiak-Markowicz I, Hildering KS, Glaser W, et al. Conventional dendritic cells mount a type I IFN response against *Candida* spp. requiring novel phagosomal TLR7-mediated IFN-beta signaling. *J Immunol*. 2011 Mar 1;186(5):3104-12.
 - 71 Bonifazi P, D'Angelo C, Zagarella S, Zelante T, Bozza S, De Luca A, et al. Intranasally delivered siRNA targeting PI3K/Akt/mTOR inflammatory pathways protects from aspergillosis. *Mucosal Immunol*. 2010 Mar;3(2):193-205.
 - 72 Fei M, Bhatia S, Oriss TB, Yarlagadda M, Khare A, Akira S, et al. TNF-alpha from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc Natl Acad Sci U S A*. 2011 Mar 29;108(13):5360-5.
 - 73 Ramirez-Ortiz ZG, Lee CK, Wang JP, Boon L, Specht CA, Levitz SM. A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. *Cell Host Microbe*. 2011 May 19;9(5):415-24.
 - 74 LeBlanc DM, Barousse MM, Fidel PL, Jr. Role for dendritic cells in immunoregulation during experimental vaginal candidiasis. *Infect Immun*. 2006 Jun;74(6):3213-21.
 - 75 van de Veerdonk FL, Marijnissen RJ, Kullberg BJ, Koenen HJ, Cheng SC, Joosten I, et al. The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe*. 2009 Apr 23;5(4):329-40.
 - 76 Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med*. 2009 Feb 16;206(2):299-311.
 - 77 Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature*. 2007 Aug 30;448(7157):1058-62.
 - 78 van de Veerdonk FL, Plantinga TS, Hoischen A, Smeekens SP, Joosten LA, Gilissen C, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. *N Engl J Med*. 2011 Jul 7;365(1):54-61.
 - 79 Gladiator A, Wangler N, Trautwein-Weidner K, LeibundGut-Landmann S. Cutting edge: IL-17-secreting innate lymphoid cells are essential for host defense against fungal infection. *J Immunol*. 2013 Jan 15;190(2):521-5.
 - 80 Mear JB, Gosset P, Kipnis E, Faure E, Dessein R, Jawhara S, et al. *Candida albicans* airway exposure primes the lung innate immune response against *Pseudomonas aeruginosa* infection through innate lymphoid cell recruitment and interleukin-22-associated mucosal response. *Infect Immun*. 2014 Jan;82(1):306-15.
 - 81 Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13

- in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med*. 2013 Aug 15;188(4):432-9.
- 82 Albacker LA, Chaudhary V, Chang YJ, Kim HY, Chuang YT, Pichavant M, et al. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nat Med*. 2013 Oct;19(10):1297-304.
- 83 Quintin J, Voigt J, van der Voort R, Jacobsen ID, Verschuere I, Hube B, et al. Differential role of NK cells against *Candida albicans* infection in immunocompetent or immunocompromised mice. *Eur J Immunol*. 2014 May 6.
- 84 Voigt J, Hunniger K, Bouzani M, Jacobsen ID, Barz D, Hube B, et al. Human natural killer cells acting as phagocytes against *Candida albicans* and mounting an inflammatory response that modulates neutrophil antifungal activity. *J Infect Dis*. 2014 Feb 15;209(4):616-26.
- 85 Schmidt S, Tramsen L, Hanisch M, Latge JP, Huenecke S, Koehl U, et al. Human natural killer cells exhibit direct activity against *Aspergillus fumigatus* hyphae, but not against resting conidia. *J Infect Dis*. 2011 Feb 1;203(3):430-5.
- 86 Bouzani M, Ok M, McCormick A, Ebel F, Kurzai O, Morton CO, et al. Human NK cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-gamma release. *J Immunol*. 2011 Aug 1;187(3):1369-76.
- 87 Park SJ, Hughes MA, Burdick M, Strieter RM, Mehrad B. Early NK cell-derived IFN- γ is essential to host defense in neutropenic invasive aspergillosis. *J Immunol*. 2009 Apr 1;182(7):4306-12.
- 88 Robert R, Nail S, Marot-Leblond A, Cottin J, Miegerville M, Quenouillere S, et al. Adherence of platelets to *Candida* species in vivo. *Infect Immun*. 2000 Feb;68(2):570-6.
- 89 Drago L, Bortolin M, Vassena C, Taschieri S, Del Fabbro M. Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity. *BMC Microbiol*. 2013;13:47.
- 90 Rodland EK, Ueland T, Pedersen TM, Halvorsen B, Muller F, Aukrust P, et al. Activation of platelets by *Aspergillus fumigatus* and potential role of platelets in the immunopathogenesis of Aspergillosis. *Infect Immun*. 2010 Mar;78(3):1269-75.
- 91 Speth C, Hagleitner M, Ott HW, Wurzner R, Lass-Flörl C, Rambach G. *Aspergillus fumigatus* activates thrombocytes by secretion of soluble compounds. *J Infect Dis*. 2013 Mar 1;207(5):823-33.
- 92 Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe*. 2012 Aug 16;12(2):223-32.
- 93 Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, et al. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clin Vaccine Immunol*. 2014 Apr;21(4):534-45.

9

Summary and perspectives



Despite the increased knowledge on the mechanisms of *Candida* recognition and the networks of innate and adaptive host defense activated during infection (1), much remains to be learned regarding the distinctive modulatory effects of *Candida spp* on host immune responses, while further studies are warranted in order to decipher the complex host-pathogens interactions and the relationship and synergy among the different PRRs.

In **Chapter 2** of this thesis we analyzed the capacity of *C. albicans* to modulate the cytokine responses of PBMCs to bacteria that normally colonize the skin and mucosa. We showed that *C. albicans* primes the cells for enhanced proinflammatory cytokine production when they are exposed to a secondary bacterial stimulus. This priming effect is dependent on β -glucan and N-linked mannan present on the *Candida* cell wall. Therefore, the chronic exposure of primary human immune cells to *C. albicans* primes them for subsequent stimulation with different microorganisms, mechanism that could explain the potential stimulatory effects of the fungus in hyperinflammatory conditions (e.g. the suggested role of *Candida* in Crohn's disease). Therefore, the Dectin-1/Raf-1 signaling pathway described to be responsible for this effect may represent a potential therapeutic target in autoinflammatory diseases.

In the next two chapters we investigated the role of Dectin-2, one important C-type lectin receptor, for the innate host defense against two of the most important *Candida* species: *C. albicans* and *C. glabrata*. In **Chapter 3** we confirmed that Dectin-2^{-/-} mice were more susceptible to systemic candidiasis, which is most probably due to the elevated fungal load in the kidneys. Phagocytosis of *C. albicans* by the Dectin-2^{-/-} macrophages was moderately decreased. Interestingly, the secretion of most of the macrophage-derived cytokines from Dectin-2^{-/-} mice with systemic candidiasis was defective. No striking differences among several *Candida* mutants defective in mannans could be detected between naïve wild-type and Dectin-2^{-/-} mice, apart from the β -mannan-deficient *bmt1 Δ /bmt2/bmt5 Δ* triple mutant, suggesting that β -mannan may partially mask α -mannan detection, which is the major fungal structure recognized by Dectin-2.

In **Chapter 4** we showed that Dectin-2 is an important component of the host defense against systemic infection with *C. glabrata*. Dectin-2^{-/-} mice were more susceptible to *C. glabrata*, susceptibility that was associated with a combination of decreased production of protective T-helper-derived cytokines and defective phagocytosis and killing of the yeast by neutrophils and macrophages, respectively. These results not only broaden our knowledge regarding the interaction between *C. glabrata* and the immune system, but they also give us insights into the possibility of designing future immunotherapeutic strategies based on Dectin-2 receptor.

Trained immunity is a novel described phenomenon through which cells associated with the innate immune system exhibit memory characteristics upon a second encounter with a microorganism or with a microbial ligand (2, 3). In **Chapter 5** we showed that a primary exposure to microbial ligands alters the functional fate of monocytes towards tolerance or training, and this is dependent on the nature of PRRs engaged and on their concentration. Trained immunity and tolerance may have important effects on the host susceptibility to infections by inducing long-term changes in innate cells, such as monocytes, macrophages or NK cells, in the capacity

of non-specifically responding to a secondary infection. This could be of exceptional interest in developing new therapeutic strategies against excessive autoinflammatory responses or for improving ineffective immune responses against an infection, by harnessing innate immune cells and their related receptors and signaling pathways.

In **Chapter 6** we investigated whether other commensal fungi, such as *Saccharomyces cerevisiae*, are also able to induce trained immunity. We show that different strains of *Saccharomyces* are able to train human primary cells, with this training effect being dependent not only on β -glucan and mannan but also on chitin. Strains specifically isolated from CD patients are able to modulate cytokines response to bacterial antigens and fungi and this effect is mostly dependent on chitin. We observed a strong strain-specific variation of the cytokine production after stimulation of monocytes with different human environmental isolates of *S. cerevisiae*, which also correlated with the origin of the strain, suggesting that that differential adaptation to the environment might be a determining factor of fungal pathogenicity.

Both patients with chronic mucocutaneous candidiasis (CMC) and patients with Hyper-IgE syndrome (HIES) have defects in generation of Th17 responses, however their phenotype in terms of types of infections differs significantly. Recently STAT1 was identified as one of the transcription factors modulated during induction of trained immunity (4), and we have thus hypothesized that defects in trained immunity might characterize patients with defects in STAT1. In **Chapter 7** we report that patients with STAT1-defective CMC are less able to mount trained immunity against *C. albicans*, and no such defect is observed in patients with STAT3-defective (HIES). These results may highlight a clinically significant fact with regard to the phenotype of those two groups of patients and their susceptibility to infections, since CMC patients predominantly suffer of *Candida* infections (5), while HIES patients encounter mainly *Staphylococcal* infections (6, 7). These findings are biologically important due to the fact that they identify a new STAT1- and NK/IFN- γ -dependent pathway that is important for induction of trained immunity.

Future perspectives

The results reported in these studies both contribute to an improved understanding of the innate immune responses activated during systemic *Candida* infections, and, at the same time, open new avenues of investigation for the future.

Candida-induced priming of innate immunity and the signaling pathways involved in this process may represent an important aspect of novel immunotherapeutic strategies, and therefore its potential exploitation in different *Candida* infections could be of significant interest, especially for patients suffering of autoinflammatory diseases. Of specific interest is the role of Dectin-2 in interaction with different *Candida* species and its synergy with other C-type lectins or with TLRs. Whether the Dectin-2 is involved in either the stimulation of Th17 immune responses or in induction and/or inhibition of innate immune cytokines may represent a new research line in the near future.

Vaccination represents the most effective manipulation of the immune system; therefore, harnessing not only the adaptive, but also the innate immune responses may lead to more efficient ways of protection against different infectious factors. Several interesting points regarding the trained immunity remain to be investigated in the coming years. What is the training effect of a broad range of different microorganisms (either commensals or pathogens) on innate immune cells and what precisely is the relationship between them? What are the distinctive mechanisms through which different ligands induce either training or tolerance? Another interesting aspect could be the effect of sequential training with the same or with different microorganisms and/or ligands? Do they still induce training/tolerance and at which level? Do the time period between the training and the restimulation influence the final outcome? Can trained immunity be reversed and if yes, what is its clinical importance?

Of specific interest remains what kind of innate immune cells, apart from monocytes/macrophages and NK cells, are able to exhibit memory characteristics. Are dendritic cells also able to respond to a secondary stimulation? What about neutrophils or mast cells? What is the synergy (if there is any) between the innate and adaptive cells in inducing trained immunity?

Up till now, several epigenetic profiles of trained monocytes and/or macrophages have been described as probably the most important molecular mechanism involved in training. In the coming years we will need to decipher the epigenetic profiles of other innate immune populations and the period during which these changes are to be found after the training.


In conclusion, the immune responses induced by *Candida* represent a complex signaling network in which *Candida* species and possibly particular strains induce specific effects, and in which cell wall morphology and the collaboration between PRRs and different cytokines play a significant role. Moreover, trained immunity represents an important newly described phenomenon that changes our understanding concerning the relation between fungi and the immune system, opening novel perspectives for therapeutic and prophylactic applications.

References

- 1 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol.* 2008 Jan;6(1):67-78.
- 2 Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe.* 2011 May 19;9(5):355-61.
- 3 Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. *Eur J Clin Invest.* 2013 Aug;43(8):881-4.
- 4 Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirofeh A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science.* 2014 Sep 26;345(6204):1251086.
- 5 Soltesz B, Toth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. *J Med Genet.* 2013 Sep;50(9):567-78.
- 6 Freeman AF, Holland SM. Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. *Pediatr Res.* 2009 May;65(5 Pt 2):32R-7R.
- 7 Paulson ML, Freeman AF, Holland SM. Hyper IgE syndrome: an update on clinical aspects and the role of signal transducer and activator of transcription 3. *Curr Opin Allergy Clin Immunol.* 2008 Dec;8(6):527-33.

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Samenvatting en perspectieven
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Samenvatting en perspectieven

Ondanks de toegenomen kennis over de mechanismen van *Candida* herkenning en de geactiveerde netwerken van het aangeboren en het adaptieve immuunsysteem tijdens de infectie (1), blijft er nog veel te leren over de onderscheidende modulerende effecten van *Candida spp* immuunreacties van de gastheer. Verdere studies zijn gerechtvaardigd om de complexe gastheer-pathogenen interacties en de synergie tussen de verschillende pathogen herkenningsreceptoren (PRRs) te ontcijferen.

In **hoofdstuk 2** van dit proefschrift, analyseerden wij de capaciteit van *C. albicans* om de cytokine productie van perifere bloed mononucleaire cellen (PBMC) op bacteriën die normaal de huid en slijmvliezen koloniseren, te moduleren. Wij hebben ontdekt dat *C. albicans* de PBMCs activeert tot een verhoogde pro-inflammatoire cytokineproductie wanneer de cellen worden blootgesteld aan een secundaire bacteriële stimulie. Dit activerend effect is afhankelijk van β -glucan en N-gekoppelde mannan die aanwezig zijn in de *Candida* celwand. Na de chronische blootstelling van primaire humane PBMCs aan *C. albicans*, worden ze geprikkeld voor een daaropvolgende stimulatie met verschillende micro-organismen. Deze mechanismen zouden de mogelijke stimulerende effecten van de schimmel in hyperinflammatoire omstandigheden (bijvoorbeeld de voorgestelde rol van *Candida* bij de ziekte van Crohn) kunnen verklaren. Daarom is de beschreven Dectin-1/Raf-1 signaalroute verantwoordelijk voor dit effect een potentieel therapeutisch doel bij autoinflammatoire ziekten.

In de volgende twee hoofdstuken, onderzochten wij de rol van Dectin-2, een van de belangrijkste C-type lectine receptoren (CLRs), voor de aangeboren afweer tegen twee belangrijke *Candida* species: *C. albicans* en *C. glabrata*. In **hoofdstuk 3**, bevestigden wij dat Dectin-2^{-/-} muizen gevoeliger zijn voor systemische candidiasis, wat waarschijnlijk veroorzaakt wordt door verhoogde fungale belasting in de nieren. De fagocytose van *C. albicans* door Dectin-2^{-/-} macrofagen is verminderd ten opzichte van wild type macrofagen. Interessant is de vinding dat cytokine productie door macrofagen van Dectin-2^{-/-} muizen met systemische candidiasis defect was. Er konden geen opvallende verschillen tussen verschillende *Candida* mutanten in mannanen worden gedetecteerd tussen naïeve wildtype en Dectin-2^{-/-} muizen, behalve de β -mannan-deficiënte *bmt1Δ/bmt2Δ/bmt5Δ* drievoudige *Candida* mutant. Deze observatie suggereert dat de β -mannan gedeeltelijk de α -mannan detectie kan maskeren en dat α -mannan de belangrijkste schimmel structuur is die door Dectin-2 herkend wordt.

In **hoofdstuk 4** hebben wij laten zien dat Dectin-2 een belangrijk onderdeel is van de afweer tegen systemische infectie met *C. glabrata*. Dectin-2^{-/-} muizen waren meer gevoelig voor *C. glabrata*; deze gevoeligheid was geassocieerd met een combinatie van de verminderde productie van beschermende T-helper-afgeleide cytokinen, een defecte fagocytose en het doden van de *Candida* door neutrofielen en macrofagen. Deze resultaten vergroten niet alleen onze kennis over de interactie tussen *C. glabrata* en het immuunsysteem, maar ze geven ons ook inzicht in de mogelijkheden van het ontwerpen van toekomstige immunotherapeutische strategieën die op de Dectin-2 receptor zijn gebaseerd.

Getrainde immuniteit is een nieuw beschreven mechanisme waardoor cellen die geassocieerd worden met het aangeboren immuunsysteem geheugenkenmerken vertonen bij een tweede ontmoeting met een micro-organisme of een microbiële bestandsdeel (2, 3). In **hoofdstuk 5**, hebben wij bewezen dat een primaire blootstelling aan microbiële liganden het functionele lot verandert van monocytten ten opzichte van tolerantie of training, en dit afhankelijk is van het betrokken type receptor en van de concentratie van de receptor (PRR). Getrainde immuniteit en tolerantie kunnen belangrijke effecten hebben op de vatbaarheid van de gastheer voor infecties. Dit zou kunnen komen door het induceren van langdurige veranderingen bij cellen van het aangeboren immuun systeem (zoals monocytten, macrofagen en NK-cellen) waardoor er een niet-specifieke reactie op treedt bij een secundaire infectie. Dit zou van uitzonderlijk belang kunnen zijn bij de ontwikkeling van nieuwe therapieën tegen overmatige autoïnfammatoire reacties, of voor het verbeteren van ineffectieve immuunrespons tegen een infectie, door gebruik te maken van aangeboren immuunsysteem cellen en hun verwante receptoren en signaalroutes.

In **hoofdstuk 6** onderzochten wij of andere commensale schimmels, zoals *Saccharomyces cerevisiae*, ook getrainde immuniteit kunnen induceren. Wij zien dat verschillende stammen van *Saccharomyces* menselijke primaire cellen (PBMCs) kunnen trainen. Dit trainingseffect is niet alleen van β -glucan en mannan afhankelijk, maar ook van chitine. Specifieke geïsoleerde *Saccharomyces* stammen van CD (Crohn) patiënten zijn in staat om de productie van cytokinen door PBMCs door bacteriële antigenen en schimmels te moduleren en dit effect is meestal afhankelijk van chitine. Wij hebben een sterke soort-specifieke variatie van de cytokine productie na stimulatie van monocytten met verschillende menselijke milieu isolaten van *S. cerevisiae* waargenomen, die ook met de oorsprong van de stam is gecorreleerd. Dit suggereert dat de differentiële aanpassing aan de omgeving een bepalende factor van schimmel pathogeniteit zou kunnen zijn.

Patiënten met chronische mucocutaneous candidiasis (CMC) of met Hyper IgE syndroom (HIES), hebben defecten in de opwikkeling van Th17 reacties, ondanks dat hun fenotype in termen van soorten infecties aanzienlijk verschillen. Onlangs werd STAT1 geïdentificeerd als een van de transcriptiefactoren die gemoduleerd is bij de inductie van getrainde immuniteit (4). Onze hypothese is dat cellen van patiënten met STAT1 defecten geen getrainde immuniteit kunnen ontwikkelen. In **hoofdstuk 7** beschreven wij dat CMC patiënten met een STAT1-defect, een defect in de opbouw van getrainde immuniteit tegen *C. albicans* hebben, en dat een dergelijk defect is niet waargenomen bij patiënten met STAT3-defect (HIES). Deze resultaten kunnen een klinisch significante waarneming benadrukken, met betrekking tot het fenotype van deze twee groepen patiënten en hun vatbaarheid voor infecties, aangezien CMC patiënten voornamelijk lijden aan *Candida* infecties (5), terwijl HIES patiënten vooral aan *Staphylococcus*-infecties lijden (6, 7). Deze resultaten zijn belangrijk vanwege het feit dat nieuwe STAT1- en NK/IFN γ -afhankelijke routes geïdentificeerd zijn die belangrijk zijn voor de inductie van getrainde immuniteit.

Toekomstperspectieven

De gerapporteerde resultaten van deze studies dragen bij aan een beter begrip van de aangeboren immuunrespons veroorzaakt door voortdurende systemische *Candida*-blootstelling?, en tegelijkertijd, bieden zij nieuwe mogelijkheden voor het onderzoek in de nabije toekomst.

Candida-geïnduceerde activering van de aangeboren immuniteit en de signaalroutes die bij dit proces betrokken zijn, zouden een belangrijk aspect van de nieuwe immunotherapeutische strategieën kunnen zijn. Van bijzonder belang is de rol van Dectin-2 in de interactie met verschillende *Candida*-soorten en haar synergie met andere C-type lectines of met TLR's. Of de Dectin-2 wordt betrokken bij de stimulatie Th17 immuunreacties of bij de inductie en/of remming van het aangeboren immuunsysteem van cytokines zou in de nabije toekomst kunnen worden onderzocht.

Vaccinatie is de meest efficiënte manipulatie van het immuunsysteem; daarom kan het gebruik van niet alleen adaptieve, maar ook van de aangeboren immuunrespons, leiden tot meer effectieve manieren van bescherming tegen diverse besmettelijke infectieziekten. Diverse interessante punten met betrekking tot de getrainde immuniteit blijven over om nader te worden onderzocht in de komende jaren. Wat is het trainingseffect van een breed scala van verschillende micro-organismen (het zij commensalen of pathogenen) op de cellen van het aangeboren immuunsysteem en wat is de preciese relatie tussen hen? Wat zijn de belangrijkste mechanismen die de verschillende microbiele liganden induceren bij training of tolerantie? Een ander interessant aspect zou kunnen zijn, is het effect van opeenvolgende training met dezelfde of met verschillende micro-organismen en/of liganden? Induceren ze nog steeds de training/tolerantie en op welk niveau? Is de tijd tussen de training en restimulatie van invloed op het uiteindelijke resultaat? Kan de getrainde immuniteit teruggedraaid worden en, zo ja, wat is het klinische belang hiervan?

Van specifiek belang blijft welke soort aangeboren immuuncellen, afgezien van monocyten/macrofagen en NK-cellen, in staat zijn om kenmerkende herinneringen te vertonen. Kunnen dendritische cellen ook reageren op een secundaire stimulatie? Hoe zit het met neutrofielen of mestcellen? Wat is de synergie (als die er is) tussen de aangeboren en adaptieve cellen tijdens het induceren van getrainde immuniteit?

Tot nu toe, zijn er verschillende epigenetische profielen van getrainde monocyten en/of macrofagen beschreven als waarschijnlijk de belangrijkste moleculaire mechanismen die betrokken zijn bij de training. Wij zullen de komende jaren nodig hebben om de epigenetische profielen van andere aangeboren immuuncellen en de periode waarin deze wijzigingen zijn terug te vinden na de training, te ontcijferen.

Concluderend, de geïnduceerde immuunreacties door *Candida* vormen een complex signalerend netwerk waarin *Candida* soorten en misschien zelfs bepaalde stammen specifieke effecten induceren en waarbij celwand morfologie en de samenwerking tussen PRRs en verschillende cytokines een belangrijke rol spelen. Bovendien is getrainde immuniteit een belangrijk nieuw beschreven mechanisme dat onze kennis van de relatie tussen schimmels en het immuunsysteem verandert. Dit opent nieuwe perspectieven voor therapeutische en profylactische toepassingen van getrainde immuniteit.

Referenties

- 1 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol*. 2008 Jan;6(1):67-78.
- 2 Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe*. 2011 May 19;9(5):355-61.
- 3 Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. *Eur J Clin Invest*. 2013 Aug;43(8):881-4.
- 4 Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajani-Refah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014 Sep 26;345(6204):1251086.
- 5 Soltesz B, Toth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. *J Med Genet*. 2013 Sep;50(9):567-78.
- 6 Freeman AF, Holland SM. Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. *Pediatr Res*. 2009 May;65(5 Pt 2):32R-7R.
- 7 Paulson ML, Freeman AF, Holland SM. Hyper IgE syndrome: an update on clinical aspects and the role of signal transducer and activator of transcription 3. *Curr Opin Allergy Clin Immunol*. 2008 Dec;8(6):527-33.

Rezumat și perspective

Deși cercetarea a avansat foarte mult în ultimii ani în ceea ce privește mecanismele de recunoaștere a *Candidaei* și activarea sistemului imunitar innăscut (nespecific) și adaptiv (specific) în timpul infecției (1), multe rămân însă neelucidate în ceea ce privește efectele modulatoare distinctive ale diferitelor specii de *Candida* și răspunsurile imunitare ale gazdei. Studii suplimentare sunt necesare în scopul de a descifra interacțiunile complexe dintre gazdă și agenții patogeni, dar și relația de sinergie dintre diferiți receptori prezenți la suprafața sau în interiorul celulelor umane.

În **capitolul 2** am analizat capacitatea *Candidaei albicans* de a modula răspunsurile citokinelor produse de celulele mononucleare din sângele uman periferic (PBMC), la bacterii care în mod normal colonizează pielea și mucoasele. Am demonstrat că ciuperca *C. albicans* stimulează celulele imune umane către o producție mărită de citokine proinflamatorii atunci când celulele sunt expuse la un stimul bacterian secundar. Acest efect este dependent de β -glucan și de N-manan prezent în peretele celular al *Candidaei*. Prin urmare, expunerea cronică a celulelor imune primare la *C. albicans* induce un efect de activare mărită către stimularea ulterioară cu diferite microorganisme, mecanism care ar putea explica eventualele efecte stimulatorie ale ciupercilor în condiții de hiperinflamare (cum ar fi rolul sugerat de *Candida* în boala Crohn). În concluzie, calea de semnalare Dectin-1/Raf-1 descrisă în acest studiu ar putea fi responsabilă pentru acest efect, reprezentând o potențială țintă terapeutică în bolile autoinflamatorii.

În următoarele două capitole am investigat rolul receptorului Dectin-2 în imunitatea innăscută împotriva a doua dintre cele mai importante specii de *Candida*: *C. albicans* și *C. glabrata*. În **capitolul 3** am confirmat faptul că șoarecii deficienți în Dectin-2 sunt mai sensibili la candidoza sistemică, aceasta datorându-se faptului că numărul coloniilor de *Candida* a fost mărit în rinichii animalelor modificate genetic (fara receptorul Dectin-2) comparativ cu animalele din grupul control (cu receptorul Dectin-2). Fagocitoza *Candidaei* de către macrofagele deficiente în Dectin-2 a fost moderat scăzută. Interesant, și secreția majorității citokinelor produse de macrofagele de la șoarecii deficienți în Dectin-2 ce sufereau de candidoză sistemică a fost deficientă. Nu există însă diferențe semnificative între mutanți ai *Candidaei* deficienți în mannan în stimularea de citokine de la ambele grupuri de șoareci: control și fără Dectin-2. Excepție este însă mutantul triplu deficient în β -manan, *bmt1Δ/bmt2Δ/bmt5Δ*, care nu mai este capabil să inducă IL-6 și KC în animalele deficiente în Dectin-2 comparativ cu controalele, sugerând că β -mannan poate masca parțial detectarea α -mananului, structura fungică majoră recunoscută de Dectin-2.

În **capitolul 4** am arătat că receptorul Dectin-2 joacă un rol important în protejarea împotriva infecțiilor sistemice cu *C. glabrata*. Șoarecii deficienți în Dectin-2 au fost mai vulnerabili la *C. glabrata*, susceptibilitate care a fost asociată cu o secreție scăzută de citokine protectoare derivate de la celulele Th, și o fagocitoză defectuoasă a *Candidaei* de către neutrofile și macrofage. Aceste rezultate ajută nu numai la extinderea cunoștințelor noastre în ceea ce

privește interacțiunea dintre *C. glabrata* și sistemul imunitar uman, dar aduce și perspective noi în ceea ce privește îmbunătățirea strategiilor imunoterapeutice bazate pe acest receptor.

Imunitatea antrenată (instruită) este fenomenul prin care celulele asociate cu sistemul imunitar înăscut (nespecific) au caracteristici de a memora prezența unui stimul la a doua interacțiune cu un microorganism sau cu un ligand de origine microbială (2, 3). În **capitolul 5** am arătat că o expunere primară a celulelor imune umane la liganzi microbiali modifică soarta funcțională a monocitelor către toleranță sau stimulare, depinzând atât de natura receptorilor implicați în acest proces, cât și de concentrația lor. Imunitatea antrenată și toleranța pot avea efecte importante asupra susceptibilității gazdei la diferite infecții prin inducerea de modificări epigenetice pe termen lung în celulele imunitare înăscute (monocite, macrofage sau celule ucigașe naturale (celulele NK)), în ceea ce privește răspunderea non-specifică la o infecție secundară. Această descoperire poate fi de interes excepțional atât în dezvoltarea de noi strategii terapeutice împotriva răspunsurilor autoinflamatorii excesive, cât și pentru îmbunătățirea răspunsurilor imune împotriva unei infecții prin valorificarea celulelor imune înăscute, a receptorilor și a căilor lor de semnalizare.

În **capitolul 6** am investigat posibilitatea altor ciuperci comensale, cum ar fi *Saccharomyces cerevisiae*, de a avea capacitatea de inducere a imunității antrenate. Interesant, am arătat că diferite tulpini de *Saccharomyces* sunt capabile de a instrui celule primare umane, efect dependent nu numai de β -glucan și manan, dar și de chitină. Tulpinile izolate în special de la pacienții cu Crohn au capacitatea de a modula răspunsul de citokine la antigene bacteriene și ciuperci, efect ce este în mare parte dependent de chitină. Am observat o corelație puternică între citokine și originea tulpinilor de *Saccharomyces* sugerând că adaptarea diferențiată la mediu ar putea fi un factor determinant al patogenității fungice.

Atât pacienții cu candidoză cronică muco-cutanată (CMC, STAT1), cât și pacienții cu sindromul Hyper-IgE (SHIE, STAT3) au defecte în răspunsurile Th17, însă fenotipul lor în ceea ce privește tipurile de infecții microbiene diferă semnificativ. Recent, STAT1 a fost identificat ca unul dintre factorii de transcripție modulat în timpul inducerii imunității antrenate (4). Ipoteza noastră s-a bazat pe faptul că defecte în imunitatea antrenată poate caracteriza pacienții cu defecte în STAT1. Prin urmare, în **capitolul 7** am raportat că pacienții cu defecte în STAT1 (CMC) sunt mai puțin capabili de a dezvolta o imunitate antrenată împotriva *Candida albicans*, defect ce nu se observă la pacienții cu sindromul SHIE (STAT3). Aceste rezultate pot evidenția un fapt clinic semnificativ în ceea ce privește fenotipul acestor două grupuri de pacienți și sensibilitatea lor la infecții, deoarece pacienții CMC suferă predominant de infecții cu *Candida* (5), în timp ce la pacienții cu sindromul SHIE persistă infecțiile stafilococice (6, 7). Aceste concluzii au importanță biologică deosebită datorită faptului că am identificat calea de semnalare STAT1- și NK/IFN- γ ca fiind importantă pentru inducerea imunității antrenate.

Perspective de viitor

Rezultatele raportate în aceste studii contribuie la o mai bună înțelegere a răspunsurilor imune înnăscute activate în timpul infecțiilor sistemice cu *Candida* și deschide perspective noi de investigare în ceea ce privește candidozele umane.

Capacitatea *Candidaei* de a antrena sistemul imun înnăscut și căile de semnalizare implicate în acest process reprezintă un aspect important în ceea ce privește dezvoltarea de noi strategii imunoterapeutice. Prin urmare, potențialul de exploatare a diferitelor infecții produse de *Candida* ar putea fi de un interes semnificativ în special pentru pacienții care suferă de boli autoinflamatorii. Interesant este și rolul receptorului Dectin-2 și interacțiunea sa cu diferite specii de *Candida*, dar și sinergia sa cu alți receptori. O nouă linie de cercetare în viitorul apropiat ar putea fi reprezentată de implicarea receptorului Dectin-2 în stimularea răspunsurilor Th17 și în stimularea și/sau inhibarea citokinelor produse de sistemul imunitar înnăscut.

Vaccinarea reprezintă cea mai eficientă manieră eficientă de manipulare a sistemului imunitar; prin urmare, valorificarea nu numai la nivel adaptiv, dar și la nivel înnăscut poate duce la modalități mai eficiente de protecție împotriva diferiților factori infecțioși (ciuperci, bacterii, virusuri etc). Multe aspecte de mare interes rămân a fi investigate în anii următori în ceea ce privește imunitatea antrenată. Care este efectul de antrenare a celulelor sistemului imunitar înnăscut de către o mare varietate de microorganisme (comensale sau patogene) și care este relația dintre ele? Care sunt mecanismele distinctive prin care diferiți liganzi induc antrenare sau toleranță? Un alt aspect interesant ar fi efectul de antrenare secvențială cu aceleași sau cu diferite tipuri de microorganisme și/sau liganzi? Ar mai putea induce antrenare/toleranță și, dacă da, la ce nivel? Va fi rezultatul final influențat de perioada de timp dintre antrenare și restimulare? Poate imunitatea antrenată fi reversibilă și, dacă da, care ar fi importanța sa clinică? Care dintre celulele sistemului imunitar înnăscut, cu excepția monocitelor/macrofagelor și a celulelor ucigașe naturale, sunt capabile să prezinte caracteristici de memorare? Sunt celulele dendritice capabile să răspundă la o stimulare secundară? Dar neutrofile sau mastocite? Ce sinergie ar fi (dacă există) între celulele înnăscute și adaptive în inducerea imunității antrenate?

Până în prezent au fost descrise mai multe profiluri epigenetice de monocite și macrofage antrenate ca fiind probabil cel mai important mecanism molecular implicat în formarea imunității antrenate. În anii următori va trebui să descifrăm atât profilurile epigenetice ale diferitelor populații de celule imune nespecifice cât și perioada în care aceste modificări se regăsesc după formare.

În concluzie, răspunsurile imune induse de *Candida* reprezintă o rețea complexă de semnalizare în care diferite specii de *Candida* și, probabil diferite tulpini, induc efecte specifice, iar morfologia peretelui celular și colaborarea dintre receptori și citokine joacă un rol foarte important. Chiar mai mult, imunitatea antrenată reprezintă un fenomen important ce revoluționează cunoștințele noastre privind relația dintre ciuperci și sistemul imunitar înnăscut, deschizând perspective noi pentru aplicații terapeutice și profilactice.

Referințe

- 1 Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol*. 2008 Jan;6(1):67-78.
- 2 Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe*. 2011 May 19;9(5):355-61.
- 3 Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. *Eur J Clin Invest*. 2013 Aug;43(8):881-4.
- 4 Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajani N, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014 Sep 26;345(6204):1251086.
- 5 Soltesz B, Toth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S, et al. New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe. *J Med Genet*. 2013 Sep;50(9):567-78.
- 6 Freeman AF, Holland SM. Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. *Pediatr Res*. 2009 May;65(5 Pt 2):32R-7R.
- 7 Paulson ML, Freeman AF, Holland SM. Hyper IgE syndrome: an update on clinical aspects and the role of signal transducer and activator of transcription 3. *Curr Opin Allergy Clin Immunol*. 2008 Dec;8(6):527-33.

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List of publications

Defective trained immunity in patients with STAT1-dependent chronic mucocutaneous candidiasis.

Ifrim DC, Quintin J, Meerstein-Kessel L, Plantinga T, Joosten LAB, van der Meer JW, van der Veerdonk F, Netea MG.
Clin Exp Immunol. 2015 Sep;181(3):434-40.

Antifungal innate immunity: recognition and inflammatory networks.

Becker KL, Ifrim DC, Quintin J, Netea MG, van de Veerdonk FL.
Semin Immunopathol. 2015 Mar;37(2):107-16.

mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity.

Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, Giamarellos-Bourboulis EJ, Martens JH, Rao NA, Aghajani-Refah A, Manjeri GR, Li Y, Ifrim DC, Arts RJ, van der Meer BM, Deen PM, Logie C, O'Neill LA, Willems P, van de Veerdonk FL, van der Meer JW, Ng A, Joosten LA, Wijmenga C, Stunnenberg HG, Xavier RJ, Netea MG.
Science. 2014 Sep 26;345(6204):1250684.

The functional fate of human monocytes after engagement of various pattern recognition receptors.

Ifrim DC, Quintin J, Joosten LAB, Jacobs L, Jansen T, Gow NAR, Williams DL, van der Meer JW, Netea MG.
Clinical and Vaccine Immunology. 2014 Apr;21(4):534-45.

Role of Dectin-2 for host defense against systemic infection with *Candida glabrata*.

Ifrim DC, Bain JM, Reid DM, Oosting M, Verschuere I, Gow NA, van Krieken JH, Brown GD, Kullberg BJ, Joosten LA, van der Meer JW, Koentgen F, Erwig LP, Quintin J, Netea MG.
Infection and Immunity. 2014 Mar;82(3):1064-73.

***Candida albicans* primes the Toll-like receptor cytokine responses through a dectin-1/Raf-1-mediated pathway.**

Ifrim DC, Joosten LAB, Kullberg BJ, Jacobs L, Jansen T, Williams DL, Gow NAR, van der Meer JW, Netea MG, Quintin J.
The Journal of Immunology. 2013 Apr 15;190(8):4129-35.

Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.

Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JW, van Crevel R, Netea MG.
Proceedings of the National Academy of Sciences USA. 2012 Oct 23;109(43):17537-42.

***Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes.**

Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg BJ, Wijmenga C, Joosten LA, Xavier RJ, van der Meer JW, Stunnenberg HG, Netea MG.
Cell Host & Microbe. 2012 Aug 16;12(2):223-32.

Fungal chitin induces trained immunity in human monocytes during cross-talk of *S. cerevisiae* with microbiota.

Rizzetto L, Ifrim DC,* Tocci N, Cheng SC, Quintin J, De Filippo C, Santos M.A.S., Netea MG, Cavalieri D.
In Press

The role of Dectin-2 for host defense against disseminated candidiasis.

Ifrim DC, Quintin J, Courjol F, Verschueren I, van Krieken JH, Koentgen F, Fradin C, Gow N.A.R., Joosten LA, van der Meer JW, van de Veerdonk F, Netea MG.
In Press

Curriculum Vitae

Daniela Camelia Ifrim was born in Râmnicu Sărat, Romania on March 11th, 1982. In 2005 she obtained her Bachelor Degree in Biochemistry (a 4 years program) from Alexandru Ioan Cuza University of Iasi, Romania. During this period Daniela went with an Erasmus scholarship at University of Central Lancashire in Preston, UK. In 2005 Daniela performed an internship in Bioinformatics at Max-Planck Research Institute in Göttingen. Between 2006 and 2009 she enrolled in a Master course in Biotechnology at Technische Universität Hamburg-Harburg, Germany. During her Masters, Daniela performed an internship at Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht in Teltow, Berlin, under the supervision of Dr Karola Lützwow, investigating the biocompatibility of biopolymeric scaffolds employed in tissue engineering. Additionally, Daniela performed a six months internship working on a project related with the development and optimization of a biochemical homogeneous assay for protein detection, at Roche Diagnostic GmbH, in Penzberg (Germany) under the supervision of Dr. Hans-Willi Krell. Furthermore, she carried on with her Master thesis working on the isolation and expansion of antigen specific human single B cells for generation of antibodies in Cell-Free Expression Systems. The research included in her thesis lasted for 9 months and was conducted by Dr Hans-Willi Krell and Dr Alexander Liefke at Roche Diagnostics GmbH in Penzberg, Germany. Daniela obtained her Master's diploma in March 2009 with a grade of very good.

Between April 2009 and July 2010 Daniela joined the Institut National de la Recherche Agronomique, laboratory of genetics and molecular microbiology in Grignon, France. The project focused on contribution of GPI-anchored proteins to the virulence of *Candida albicans* and was part of FINSysB Initial Training network. In September 2010, Daniela started her PhD at the Internal Medicine Department at Radboudumc with Prof. Mihai G. Netea and Prof. Leo A.B. Joosten as promotors and Dr. Jessica Quintin as copromotor. The aim of the PhD thesis was to study the recognition of *Candida* by C-type lectin receptors, with emphasizes on Dectin-2 receptor, as well as trained immunity phenomenon. During her PhD Daniela C. Ifrim took part in the ALLFUN European training network (Fungi in the setting of inflammation, allergy and autoimmune diseases: Translating basic science into clinical practices) and went to several national and international conferences where she presented her work.

After her PhD, Daniela took several courses in Clinical Trials and Project Management in Clinical Trials at Pharmaschool UK.

In January 2012 Daniela met Robbert van 't Ent with whom she married in August 2014. Their son, Rafael, was born on 12th of March 2014.

